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**THE ROLES OF DIFFERENT ADIPOSE DEPOTS IN
GLUTAMINE METABOLISM FOLLOWING FEEDING,
FASTING AND EXERCISE IN THE GUINEA-PIG**

A thesis submitted to the Open University in candidature
for the degree of Doctor of Philosophy

by

Janet Elizabeth Digby

December 1997

Full name of candidate: _____
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In loving memory of my father

FOREWORD

The studies presented in this dissertation were performed in the Department of Biology, The Open University, Walton Hall, Milton Keynes, between January 1993 and April 1996. Except where stated all the work described is my own. No part of this dissertation has been or is being submitted for a degree at any other University. The length of this dissertation lies within the word limit set by the Research Degrees Centre.

Janet Elizabeth Digby

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADP	adenosine 5'-diphosphate
ASP	acylation stimulating protein
ATP	adenosine 5'-triphosphate
BCAA	branch-chain amino acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CRE	cAMP response elements
EDTA	ethylenediaminetetra-acetic acid
GIDH	glutamate dehydrogenase
GRE	glucocorticoid response element
HK	hexokinase
HSL	hormone-sensitive lipase
K _m	Michaelis constant
KRB	Krebs-ringer bicarbonate buffer
LPL	lipoprotein lipase
NA	noradrenaline
NEFA	non-esterified fatty acids
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
PBS	phosphate buffered saline
PFK	phosphofructokinase
TRA	triethanolamine

ABSTRACT

This study investigates the activities of glutaminase and glutamine synthetase the two principle enzymes of glutamine metabolism, over a time course, in adipose tissue from eleven different sites of adult male guinea-pigs after feeding, fasting and exercise. In addition, the uptake and release of glutamine was measured in isolated adipocytes from five of these adipose sites in the presence of insulin, or the synthetic hormone analogues dexamethasone and isoprenaline.

In the fasted condition, glutaminase and glutamine synthetase activities were greatest in adipose tissue from the subcutaneous inguinal depot and the intra-abdominal omental depot. In the early post-prandial period, glutaminase activity was increased in adipose tissue from all sites measured, the greatest increases being observed in the omental and cervical samples. Glutamine synthetase activity was also increased in the early post-prandial period in the intra-abdominal epididymal depot and in the superficial and intermuscular depots. After exercise, glutaminase activity was decreased in adipose tissue from the inguinal, omental, perirenal, epididymal and popliteal sites. A significant effect of brief, acute exercise was observed in the intra-abdominal sites where glutamine synthetase activity was reduced compared to levels in homologous samples from the sedentary fasted guinea-pigs.

Measurements of glutamine uptake and release in isolated adipocytes prepared from different adipose depots incubated with insulin, dexamethasone and isoprenaline showed some site-specific differences that were consistent with the alterations in glutaminase and glutamine synthetase activities in homologous samples after feeding, fasting and exercise. A physiological concentration of insulin stimulated glutamine uptake into isolated adipocytes and the greatest dose-response was observed in the those prepared from the inguinal and omental sites. Glutamine uptake into isolated adipocytes from all depots studied was unaffected by dexamethasone. With isoprenaline, glutamine uptake was lower in adipocytes prepared from the inguinal, mesenteric and omental sites compared to control values. Glutamine release from omental adipocytes was greater than that from adipocytes prepared from any of the remaining sites measured. Dexamethasone enhanced glutamine release from adipocytes prepared from inguinal and epididymal adipose tissue.

These data show that adipose tissue from the relatively large superficial sites have the capacity to contribute significantly to circulating glutamine levels in the fasted and fed state and that the omental site may contribute more to the supply of glutamine to local immune tissues. The data also show that in the fed state the total adipose tissue mass of the guinea-pig may contribute to approximately 30% of the whole-body glutamine release compared to that of muscle. This conclusion is in agreement with a previous *in vivo* study of human subcutaneous adipose tissue by Frayn *et al.* (1991)

who calculated that adipose may contribute up to a third as much as that of skeletal muscle to whole-body glutamine supply.

Concomittant activity of glutaminase and glutamine synthetase activity in the fasted and fed state coupled to the capacity for adipose tissue to take up and release glutamine suggests the existence of a substrate cycle between glutamine and glutamate in adipose tissue. Such a cycle would enable efficient regulation of glutamine supply to maintain circulating levels, and, when in excess, facilitate storage of glutamine carbons by incorporation into triacylglycerols.

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Chapter 1

General Introduction

1.1 The distribution and site-specific properties of mammalian adipose tissue

Adipose tissue in adult mammals is organised in numerous discrete depots which share a blood supply and are associated with various tissues and organs including skin, skeletal and cardiac muscle, the gut, and lymph nodes (reviewed by Pond, 1996). This arrangement is in contrast to that found in many other classes of vertebrates including reptiles and amphibians which store their adipose tissue as one or a pair of large, usually intra-abdominal depots located near the centre of gravity. Why mammalian adipose tissue is partitioned into numerous depots is not clear, but may entail site-specific differences in physiological properties which could allow for local interactions with adjacent tissues and organs with which they share a blood supply (Pond 1996).

Fatty acid esterification and triacylglycerol hydrolysis may occur simultaneously within adipose tissue and it is thought that this 'futile' cycling of lipids represents a substrate cycle which enhances sensitivity in the control of fatty acid mobilisation allowing fast adaptation to changes in the rate of release of fatty acids (Newsholme and Leech 1983). Factors that are known to regulate the storage and mobilisation of lipids in adipose tissue include endocrine hormones such as insulin, catecholamines, cortisol, thyroid and growth hormone. Parahormones such as the prostaglandins and adenosine, which are produced and act locally, are also known to regulate lipolysis. Acylation Stimulating Protein (ASP) is a potent stimulant of triacylglycerol synthesis in adipocytes (Baldo *et al.* 1993) and has also been shown to stimulate glucose transport in both human preadipocytes and in fully differentiated adipocytes (Maslowska *et al.* 1997). Intra-cellular concentrations of fatty acids can also affect the biosynthesis of enzymes and proteins involved in the synthesis or breakdown and release of triacylglycerols as fatty acids themselves are believed to act as signal molecules. Local site-specific differences in the capacity of adipose depots to respond to these systemic controls along with site-specific differences in the production of secretory molecules may represent important factors determining regional adipose tissue metabolism.

Site-specific differences in adipose tissue lipid metabolism have been reported in rodents (Mattacks *et al.* 1987; Mattacks and Pond 1988; Pond and Mattacks 1991) and in humans (Östman *et al.* 1979; Arner 1995; Vikman *et al.* 1996). A summary of some site-specific properties of rodent adipose tissue depots is shown in Table 1.

Table 1.1. (See overleaf). Summary of some properties of rodent adipose tissue depots (*data summarised by Pond 1992, # data from Morimoto *et al.* 1997). Abbreviations: HK: hexokinase, PFK: phosphofructokinase, NA: noradrenaline, LPL: lipoprotein lipase, HSL: hormone-sensitive lipase, Sed: sedentary.

Table 1.1 Summary of some properties of rodent adipose tissue depots.

	Intermuscular:	Intra-abdominal: Perirenal & epididymal	Intra-abdominal: Omental & mesenteric	Superficial: Inguinal	Superficial: Shoulder & interscapular	Superficial: Behind fore-arm
*Hexokinase & Phosphofructokinase (glycolytic capacity)						
Sed/ad lib. fed	High	Low	Moderate	Low	Moderate/high	Moderate
Exercise	Unchanged	Much reduced by regular exercise	Much reduced by regular exercise	Much reduced by regular exercise	Much reduced by regular exercise	Much reduced by regular exercise
Restricted diet	Reduced	Reduced	Greatly reduced	Reduced	Reduced	Reduced
*Rates of fatty acid/TAG cycling in vivo:						
Sed/fasting	Very high	Low	Moderate	Very low	High	Moderate
Exercise	Large increase	Minimal increase	No change	Minimal increase	Large increase	Small increase
*Noradrenaline-stimulated lipolysis in vitro:						
Sed/Fasting	Less to low concentration of NA	Low	Less to low, more to high concentration	Low	More to high concentration	Low
#Hormone-sensitive lipase activity						
	Not known	High	High	Low	Not known	Not known
*Lipoprotein lipase activity in whole tissue:						
Sed/Fasting	Very high	Very low	Moderate	Low	High	Moderate
Exercise	Much reduced	Unchanged	Small reduction	Unchanged	Small reduction	Small reduction
Feeding	Unchanged	Large rise	Unchanged	Large rise	Unchanged	Large rise

In adult guinea-pigs, the subcutaneous inguinal and behind fore-arm depots and intra-abdominal perirenal depot have the highest resting rate of lipolysis and the lowest response to noradrenaline (Pond and Mattacks 1991). In rats, noradrenaline induced lipolysis and hormone-sensitive lipase activity is greater in intra-abdominal mesenteric and omental adipocytes than in the subcutaneous femoral and abdominal sites (Morimoto *et al.* 1997). Similar findings have also been reported in humans (reviewed by Bouchard *et al.* 1993) in which catecholamine-stimulated lipolysis is low in the peripheral femoral and gluteal subcutaneous adipose depots, intermediate in the abdominal subcutaneous depot and high in the intra-abdominal omental and mesenteric depots (Arner 1995; Van Harmelen *et al.* 1997). As the blood supply from the intra-abdominal mesenteric and omental depots drains into the hepatic portal vein, free fatty acids released through lipolysis are delivered directly to the liver, which may be of pathophysiological importance for the metabolic and cardiovascular complications associated with certain forms of obesity (reviewed by Kissebah and Krakower 1994).

Several mechanisms responsible for the site-specific differences in lipolysis have been proposed, including differences in the number or type of stimulatory β -adrenergic receptors or inhibitory α -adrenergic receptors (Hellmer *et al.* 1992) and regional differences in hormone-sensitive lipase activity (Morimoto *et al.* 1997) and differences in the antilipolytic effect of insulin (Bolinder *et al.* 1983). These effects may be due to adipose tissue from different anatomical sites being characterised by different patterns of gene expression and regulation which produce intrinsic site-specific differences in some aspects of adipose tissue metabolism (Cousin *et al.* 1993; Montague *et al.* 1997; Zheng *et al.* 1996).

Adipose depots that contain lymph nodes or other lymphatic tissue also show between-depot, and within-depot site-differences in rates of lipolysis in the presence of lymphocytes, and in the fatty acid composition of triacylglycerols (Pond and Mattacks 1995). In the study by Pond & Mattacks (1995), incubation with lymphoid cells significantly stimulated lipolysis in adipose explants from all sites studied except that of perirenal. In addition, adipose tissue explants incubated with lymphoid cells inhibited

mitogen-stimulated lymphocyte proliferation, and samples of adipose tissue which were taken from close to lymph nodes were significantly more effective than samples taken from sites far away from lymph nodes. It was suggested that the properties and composition of adipose tissue surrounding lymph nodes enables it to interact locally with the immune system by supplying fatty acids and possibly other substrates which may serve as fuel or regulatory molecules.

Since lipoprotein lipase (LPL) is involved in mediating fatty acid uptake into adipose tissue, it is potentially a key regulator of fat accumulation in different depots. Site-specific differences in the activity of LPL have been shown in humans (Rebuffé-Scrive 1987) and in rodents (West *et al.* 1989; Pond *et al.* 1992). In addition, site-specific differences in the regulation of LPL activity and gene expression by insulin and glucocorticoids have been reported (Fried *et al.* 1993).

Alterations in adipose tissue mass can occur either by an increase in the absolute number of adipocytes and/or by an increase in their size through lipid accumulation. The mechanisms which control the proliferation of adipocytes in particular adipose sites are poorly understood. However, selective accumulation of adipose tissue in different depots may be dependent upon the differential ability of adipocytes in these depots to proliferate and synthesise, store and release lipids. Factors which control adipose distribution may result from differing hormonal, paracrine, nutritional, circulatory and neurological milieux among adipose sites. For example, glucocorticoid receptor number has been shown to be higher in human omental compared to subcutaneous adipocytes (Rebuffé-Scrive *et al.* 1990) which may facilitate differential corticosteroid-induced regulation of adipocyte volume. The same may be true for the differential response to the catecholamines discussed previously. In addition, the higher insulin concentration in the portal circulation may contribute to differential effects of insulin on omental and mesenteric adipose depots compared to the peripheral subcutaneous depots.

Furthermore, using *in vitro* models of preadipocyte differentiation, it has been suggested that adipose tissue differentiation and accumulation in different adipose sites

may be a function of intrinsic differences in gene expression which alter the capacity to respond to factors which enhance differentiation (Kirkland *et al.* 1996).

The site-specific accumulation of adipose tissue in humans and in rodents has been studied almost entirely from the point of view of lipid or carbohydrate metabolism. However, adipose tissue also has the capacity to metabolise amino acids (discussed in Section 1.2) (Christophe *et al.* 1966; Rosenthal *et al.* 1974; Snell and Duff 1977; Frayn *et al.* 1991; Kowalski and Watford 1994a). Some site-specific differences in amino acid metabolism, specifically that of glutamine, have been reported (Kowalchuk *et al.* 1988; Kowalski and Watford 1994b) but the matter has not been studied in depth. Very little is known about the implications of adipocyte number for amino acid metabolism or whether site-specific differences in amino acid metabolism affect adipose tissue distribution. A clearer understanding of all the factors, including amino acids, which influence the underlying pattern of adipose distribution in mammals and its site-specific differences in adipose tissue metabolism will help in the understanding of how this tissue's anatomical arrangement contributes to energy and amino acid homeostasis in mammals.

1.2 Amino acid metabolism in adipose tissue

As well as its established role in lipid metabolism, adipose tissue also has the capacity to incorporate, modify and secrete amino acids, but its exact role in the metabolism of amino acids in the body as a whole is not clear. Christophe *et al.* (1966) showed that rat epididymal adipose tissue incorporated alanine, glutamate, and that it releases aspartate and glutamine. In another study by Snell and Duff (1977) rat epididymal fat pads incubated *in vitro* released alanine and the process was found to be enhanced in the presence of glucose, glutamate, leucine or valine. As this increase in release was inhibited by aminooxyacetate, it was concluded that alanine was synthesised from pyruvate transamination rather than as a result of protein breakdown in adipose tissue. These workers suggested that along with muscle, adipose tissue contributes to the production of alanine as a gluconeogenic precursor. Tischler and Goldberg (1980) showed that *in vitro* epididymal adipose tissue explants from fed rats rapidly degraded the branch

chain amino acids, leucine, isoleucine and valine to CO₂ and that this process was stimulated by glucose and insulin. This study also found that incubated rat epididymal adipose tissue released significant quantities of glutamine and lesser amounts of alanine and glutamate. Kowalchuk *et al* (1988) showed that isolated rat epididymal adipocytes utilised significant quantities of glutamine and produced glutamate and alanine. They found that the rate of glutamine utilisation and production of glutamate and alanine were enhanced by the addition of glucose and insulin to the incubation medium and the rate of incorporation of [¹⁴C] from glutamine into triacylglycerols was increased when glucose or glucose plus insulin was added to the incubation medium. Conversely, when the β -agonist isoprenaline was added with glucose to the incubation medium the rate of incorporation of [¹⁴C] glutamine into CO₂ was enhanced and the rate of its incorporation into triacylglycerol was decreased.

The exchange of alanine, glutamine and glutamate across human subcutaneous adipose tissue has also been measured *in vivo* by analysing arteriovenous differences in their concentrations before and after a meal (Frayn *et al.* 1991). This study reported that after an overnight fast, abdominal subcutaneous adipose tissue *in vivo* showed a net release of alanine and glutamine and uptake of glutamate with this pattern remaining constant after a meal except for a short period of uptake of alanine as its circulating concentration rose. Frayn *et al.* estimated that whole adipose tissue mass could contribute up to a third as much to the whole-body glutamine pool as that of skeletal muscles. Also, using *in vivo* microdialysis techniques with inguinal adipose tissue of the rat, Kowalski and Watford (1994a) reported similar findings to those of Frayn *et al.* These extrapolations however, were made from measuring only one adipose depot, chosen for their surgical accessibility rather than for their homology to depots known to have particular site-specific properties. Therefore, estimations of this tissue's contribution to the whole-body glutamine pool have been based on the assumption that all adipose depots behave in the same way. As with lipid metabolism, there may be site-specific differences in the pattern of glutamine metabolism in adipose tissue which could affect its overall net contribution to whole-body amino acid disposal and production. In particular, the fact that glutamine is

secreted by adipose tissue suggests that it may play an important role in the supply of this amino acid to other utilising tissues and organs. However very little is known about how the metabolism of glutamine in adipose tissue differs between and within depots.

Adipose tissues involvement in glutamine metabolism adds to the list of its metabolic functions and indicates that it also plays a role in amino acid as well as lipid metabolism. The study of site-specific differences in the metabolism of glutamine in adipose tissue from animals in different physiological and nutritional states will further help to characterise its contribution to whole-body glutamine homeostasis.

1.3. The structure and properties of glutamine

Glutamine is an amide derivative of the acidic amino acid glutamate. Thus it has two nitrogen-containing moieties and a polar side-chain which is a site for hydrogen bonding and enables water solubility (Figure 1.1). These properties enable this amino acid to serve as a major carrier of nitrogen as well as of carbon in the circulation between peripheral tissues and visceral organs (Welbourne 1987) and may serve similar roles at a more local level.

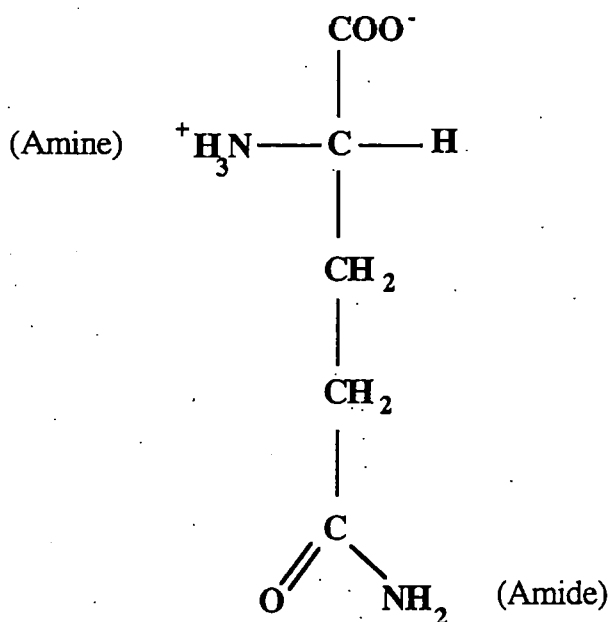


Figure 1.1. The structure of glutamine

1.4. Glutamine metabolism in mammals

As well as being a structural component of proteins, glutamine is quantitatively one of the most important amino acids in mammals, as it plays a central role in many metabolic pathways. It is the most abundant free amino acid in the mammalian circulation. In adult humans, the plasma glutamine concentration ranges between 0.6 and 0.9 mmol litre⁻¹, accounting for approximately 20% of the total plasma α -amino acids (Felig 1975). In the rat, the plasma glutamine pool turns over rapidly at a rate of approximately 130 μ mol 100 g⁻¹ body mass hour⁻¹ (Squires and Brosnan 1983) consistent with the idea that it is continuously taken up and released and so plays a major role in the transfer of carbon and nitrogen between tissues.

An overview of the role of glutamine in intermediary metabolism is shown in Figure 1.2. The amide group supplied by glutamine is used as a preferred source of nitrogen for the synthesis of N³ and N⁹ of the purine ring and, through carbamyl phosphate, N¹ of the pyrimidine ring for RNA and DNA synthesis. It also supplies the amide nitrogen atoms of asparagine and NAD as well as the amino groups of glucosamine-6-phosphate, guanine and cytidine. The α -nitrogen in glutamine can be transferred to other α -ketoacids to produce the corresponding amino acids such as alanine and serine. The carbon skeleton of glutamine can also be used as an energy substrate whereby it is partially oxidised (glutaminolysis), entering the citric acid cycle via glutamate and 2-oxoglutarate. Furthermore, ammonia produced from the catabolism of other amino acids can be incorporated into glutamine, providing an effective method of removing this potentially toxic compound from tissues.

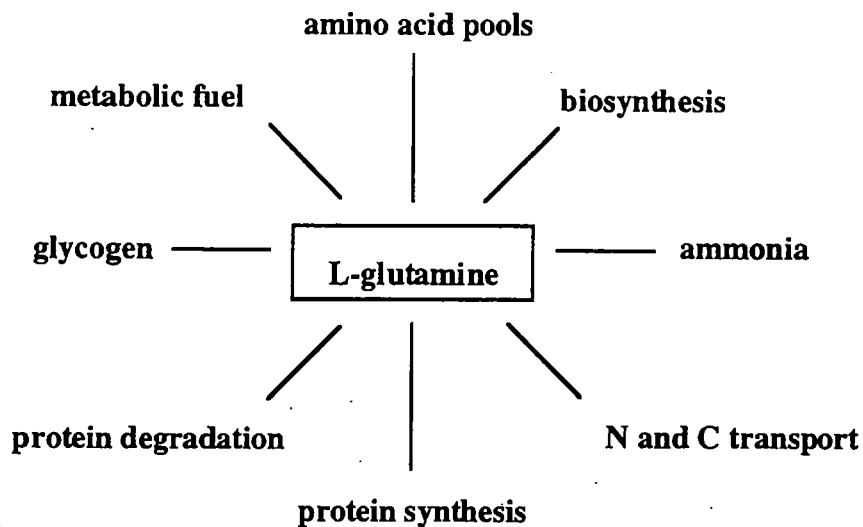


Figure 1.2. Outline of the role of glutamine in intermediary metabolism in mammalian tissues.

1.5. Overview of the catabolism of glutamine in different tissues and organs

The structure of glutamine enables a central involvement in many metabolic pathways. With metabolic acidosis, the kidney extracts significant quantities of glutamine from the circulation (Kovacevic and McGivan 1983). The amide and amine nitrogens (see Figure 1.1) of glutamine are reduced to ammonia, which is excreted in the urine, allowing excretion of anions and titratable acids without depleting sodium and potassium levels within the body. Hydrolysis of glutamine in the kidney can also provide a major precursor for kidney gluconeogenesis which is also associated with acidosis (Goodman *et al.* 1966). In the liver, glutamine is an important substrate for urea synthesis and, at concentrations greater than 10 mM, its carbon skeleton is utilised for hepatic gluconeogenesis. In post-absorptive humans, glutamine may be quantitatively more important than alanine for gluconeogenesis (Nurjhan *et al.* 1995; Perriello *et al.* 1995). The study by Perriello *et al.* demonstrated that in post-absorptive humans, the rate of appearance of glutamine in plasma was greater than that of alanine. Furthermore, since more glutamine carbon than

alanine released into plasma was derived directly from muscle proteolysis, glutamine may be quantitatively more important than alanine as a vehicle for transporting nitrogen through plasma and as a gluconeogenic precursor that adds to the plasma glucose pool.

Rapidly replicating cells, such as those associated with developing bone (Blitz *et al.* 1982), skin (Keast *et al.* 1989), hair follicles (Williams *et al.* 1993), the immune system (reviewed by Calder 1994), and mucosal cells lining the wall of the small intestine (Windmueller and Spaeth 1974) all utilise large quantities of glutamine. In these cells, nitrogen donated by glutamine can be used for the biosynthesis of purines and pyrimidines but the carbon skeleton is also the major respiratory fuel. In rats, the intestinal mucosa is thought to be a major site of glutamine disposal but, cells of the immune system also utilise significant quantities of glutamine even in the resting state (Ardawi and Newsholme 1983). Under immunological challenge, the rate of glutamine utilisation by lymphocytes is greatly increased, suggesting that it plays an important role in the metabolism of these cells. The rate of glutaminolysis in lymphocytes is markedly in excess of the actual rate of pyrimidine and purine synthesis (Szondy and Newsholme 1991). If glutamine was an important substrate for energy generation then it would be expected that more of the glutamine-derived carbon would be fully oxidised (Ardawi and Newsholme 1982). One hypothesis based on 'metabolic control logic' (Newsholme and Ardawi 1989), proposes that the high rate of glutaminolysis is important to provide optimal conditions for the precise regulation of the rate of purine and pyrimidine nucleotide synthesis at specific times of the cell cycle. Thus, a high rate of glutaminolysis would represent a control mechanism whereby synthesis of macromolecules could be regulated precisely as and when they are required.

Since glutamine can cross the blood/brain barrier, it can also serve as a precursor for the synthesis of glutamate, the most abundant free amino acid in the central nervous system and a major excitatory neurotransmitter (Meister 1984).

Adipose tissue is believed to be a consumer of glutamine as isolated rat epididymal adipocytes have been shown to utilise it either for an energy substrate or as a precursor for lipogenesis (discussed in Section 1.2) (Kowalchuk *et al.* 1988). However,

the metabolic role(s) of glutamine in this tissue are less than clear, as the main requirement for glutamine is unlikely to be involved in either of the previously mentioned functions relating to RNA or DNA synthesis and acid/base balance.

1.6. Inter-organ flows of glutamine

Glutamine is nominally designated a non-essential amino acid. However, glutamine is thought to be the principal fuel used by the intestinal tract with little or no net absorption occurring from the diet (Windmueller and Spaeth 1974; Shrock and Goldstein 1981; Felig 1975). The portally drained viscera take up more arterial glutamine than they release, indicating that the large pool of free glutamine within the body is synthesised *de novo*. It has therefore been described as a "conditionally essential" amino acid (Lacey and Wilmore 1990) that is, it becomes essential when catabolic conditions prevail. Close regulation of glutamine metabolism in different tissues is thought to be essential to the healthy function of the tissues and organs that utilise this amino acid.

Maintenance of whole-body glutamine homeostasis represents a balance between tissue and/or organ glutamine utilisation and production. *In vivo* studies have shown that skeletal muscle of rats and humans is a major site of glutamine synthesis and release into the general circulation (Rudermann and Berger 1974; Elia *et al.* 1989). In muscle, glutamine can be synthesised from any two amino acids to produce 2-oxoglutarate which is then transaminated to yield glutamate, from which a further amidation is then required to form glutamine. Branch chain amino acids (BCAA), leucine, isoleucine and valine, are thought to be the main substrates for glutamine synthesis in muscle (Chang and Goldberg 1978).

In rats, plasma glutamine depletion is often associated with trauma such as burns (Parry-Billings *et al.* 1990), major surgery (Parry-Billings *et al.* 1992), injury (Askanazi *et al.* 1980) and in the overtrained human athlete (Newsholme *et al.* 1991). As glutamine is used at a high rate by cells of the immune system, it has been suggested that the reduction in the plasma levels of this amino acid contributes to the immunosuppression observed with these conditions (Calder 1994). The hydrolysis of glutamine by the cells of the small

intestine is also increased by up to 75% following surgery (Souba 1993b). The augmented intestinal glutamine metabolism after surgical stress is thought to be stimulated by glucocorticoids (Souba *et al.* 1985). The supply of glutamine is, in part, thought to be maintained by synthesis and release from skeletal muscle as there is a concurrent increase in glutamine release from this tissue with such catabolic conditions (Ardawi and Jamal 1990a, Welbourne 1987). However, determinations of whole-body glutamine balance have suggested that net utilisation by the splanchnic bed, kidney and lymphoid tissues cannot be supplied by muscle production alone (Shrock and Goldstein 1981; Welbourne 1987). It has been proposed that in the rat, the lungs may contribute to the supply of glutamine in normal and catabolic situations (Welbourne 1988; Ardawi 1991a). However, there is some controversy as to whether this process occurs in humans, as an *in vivo* study in post-operative patients did not provide any evidence that the lungs contribute significantly to the supply of glutamine (Van Berlo *et al.* 1996).

It has recently been suggested that adipose tissue may serve as a significant source of glutamine. *In vivo* arteriovenous difference measurements in humans (Frayn *et al.* 1991) and microdialysis experiments in rats (Kowalski and Watford 1994a) indicate that adipose tissue has the capacity to release physiologically significant quantities of glutamine. An outline of the proposed interorgan flow of glutamine is shown in Figure 1.3.

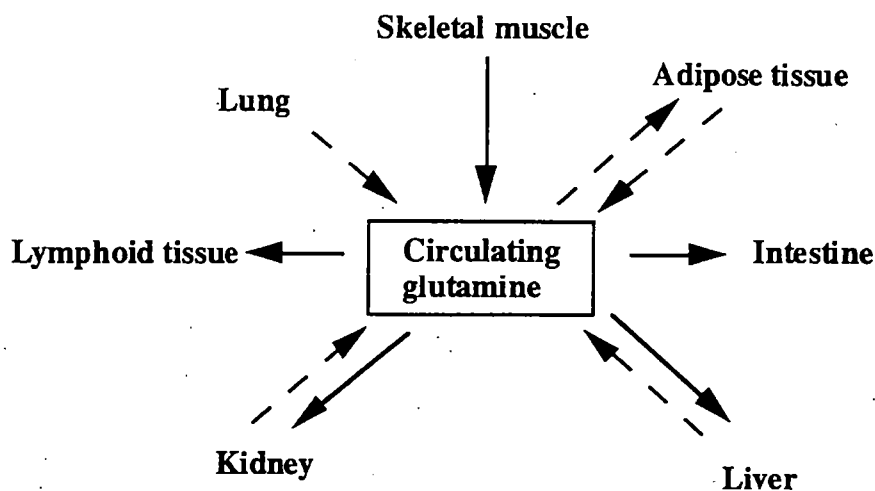
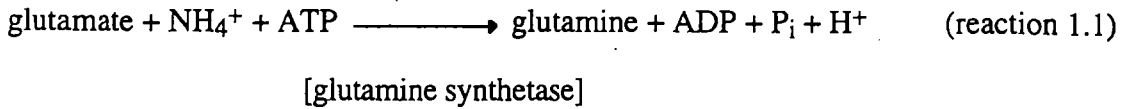


Figure 1.3. Proposed inter-organ flow of glutamine. The direction of the solid arrows represent the flow of glutamine from tissues which are thought to contribute to the supply of glutamine to utilising tissues. Arrows with broken lines indicate that it has not as yet been established whether this tissue predominantly utilises or produces glutamine and its quantitative contribution to whole-body glutamine supply.

1.7. The key enzymes of glutamine metabolism

The two key enzymes involved in the catabolism and synthesis of glutamine in mammals are glutaminase and glutamine synthetase respectively.

The synthesis and hydrolysis of glutamine in mammalian tissues were first described by Krebs in 1935. Although the exact pathway for precursor formation is not fully understood, the only known reaction yielding glutamine is that catabolised by the cytosolic enzyme glutamine synthetase (EC 6.3.1.2, L-glutamine: ammonia ligase ATP)(reaction 1.1). This enzyme catalyses the conversion of glutamate and ammonia to glutamine with the hydrolysis of ATP. A divalent cation such as magnesium is also required for catalysis: :



Glutamine synthetase is found in many tissues including brain (Pamijans *et al* 1962), liver (Meister 1984), muscle (King *et al.* 1983), lymph nodes (Ardawi and Newsholme 1984a), intestine (Ardawi and Newsholme 1988b), stomach lining (James *et al.* 1994), lung (Ardawi 1990b), kidney (Watford 1991), and epididymal and inguinal white adipose tissue (Miller 1975; Kowalski and Watford 1994b) and rat interscapular brown adipose tissue (López-Soriano *et al.* 1987).

Skeletal muscle has a relatively low glutamine synthetase activity per gram of wet tissue, but because of its large mass, is thought to be a major contributor to glutamine synthesis and release into the circulation (Rudermann and Berger 1974).

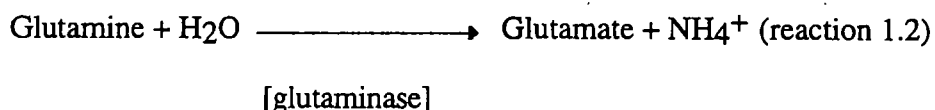
The diversity of metabolic functions served by glutamine in different tissues suggests that alterations in glutamine synthetase activity may play a key role in the provision of glutamine and therefore be subject to acute regulation. Increased utilisation of glutamine by different tissues must be balanced either by increased production or decreased utilisation at other sites within the body. Therefore, identification of tissues which synthesise and release glutamine is required to understand their quantitative contribution to the maintenance of whole-body glutamine homeostasis.

Regulation of glutamine synthetase activity *in vivo*, may, in part, be associated with alterations in the plasma levels of hormones associated with different physiological conditions. For example, maximal glutamine synthetase activity in skeletal muscle *ex vivo* is increased with conditions associated with high circulating levels of glucocorticoids such as sepsis (Ardawi and Majzoub 1991c), cancer cachexia (Souba 1993a), thermal injury (Ardawi 1988a), fasting and acidosis (King *et al.* 1983). Glutamine synthetase activity in the lungs of rats is also enhanced with various catabolic conditions including starvation, diabetes and sepsis (Ardawi 1990b).

Furthermore, with *in vivo* glucocorticoid treatment there is an increase in the maximal activity of glutamine synthetase in muscle (King *et al.* 1983), lung (Ardawi 1991b), and in mucosal cells isolated from rats made endotoxic by lipopolysaccharide treatment (Sarantos *et al.* 1994). The stimulatory effects of glucocorticoids on glutamine synthetase activity can also be modulated. In rats, endurance exercise has been shown to reduce glucocorticoid-induced glutamine synthetase activity and expression of its mRNA in fast twitch red muscle fibres. It is suggested that this process acts to conserve muscle protein by preventing breakdown of contractile proteins to release amino acids for glutamine synthesis (Falduto *et al.* 1992a and b).

Glutamine synthetase activity in cultured 3T3-L1 preadipocytes and differentiated adipocytes has also been shown to be modulated by insulin and hydrocortisone. Hydrocortisone increases, and insulin decreases, glutamine synthetase mRNA and gene transcription (Miller *et al.* 1983; Saini *et al.* 1990). However, to the author's knowledge there is no information on the effects of insulin and hydrocortisone on glutamine synthetase activity and mRNA in mature adipocytes.

Many enzymes can utilise glutamine as a substrate, but only one gives rise to stoichiometric amounts of glutamate and ammonia, that is glutaminase (reaction 1.2). Glutaminase is located exclusively in mitochondria and is thought to be associated with the inner mitochondrial membrane (Kovacevic and McGivan 1983).



There are two known isoforms, kidney-type glutaminase and liver-type glutaminase, so called after the tissues in which they were first described (Krebs 1935). Kidney-type glutaminase has also been identified in many other non-renal glutamine utilising tissues including intestinal mucosal cells, brain cells, lymphoid cells, muscle and adipose tissue (for review see Curthoys and Watford 1995). Kidney-type glutaminase and liver-type glutaminases are the products of different, but related, genes (Smith and

Watford 1990). These isoenzymes can be distinguished immunologically and have different kinetic properties and pH optima (review edited by Krebs 1980). The kidney-type glutaminase (L-glutamine aminohydrolase, EC.3.5.1.2) is also referred to as glutaminase I, phosphate-dependent glutaminase, and phosphate-activated glutaminase, as phosphate is required for maximal activation *in vitro* (Curthoys and Lowry 1973). However, as a supra-physiologic concentration of phosphate (150-100 mM) is required for maximal activation *in vitro*, it has been suggested that other physiological activators are more relevant to the *in vivo* situation. Kvamme and Torgner (1974) showed acetyl-coenzyme A (Acetyl-CoA) enhances the activating effect of phosphate on phosphate-activated glutaminase isolated from pig brain and kidney, whereas acetyl-CoA alone decreases this activation. They suggested that intracellular alterations in the concentrations of phosphate and acetyl-CoA may serve to regulate glutaminase activity *in vivo*. In a further study the effects of acyl-CoA derivatives with 5-18 fatty acyl carbons on glutaminase activity in pig brain and kidney were measured *in vitro* (Kvamme and Torgner 1975). These workers found that acyl-CoA derivatives were more effective inhibitors of glutaminase when the fatty acyl chain is elongated and more effective activators on unsaturation of the fatty acyl group. In particular, palmitoyl-CoA and stearoyl-CoA were strong inhibitors of glutaminase at a concentration of 10 μ M whereas the unsaturated fatty acyl-CoA derivatives, oleoyl-CoA and linoleoyl-CoA were potent activators at a concentration of 50 μ M. The physiological importance of the effects of fatty acyl-CoA derivatives on glutaminase activity *in vivo* is unknown, however, it may represent an example of a refined system of metabolic regulation that links ammonia production and amino acid metabolism to lipid synthesis and modification. In addition, the effect of fatty acids on glutaminase activity in adipose tissue has not previously been studied and could be of particular significance to its metabolism of glutamine.

As glutaminase is the major enzyme of glutamine catabolism (Kovacevic and McGivan 1983), its regulation in different tissues is of central importance to whole-body glutamine metabolism. Understanding the regulation of glutaminase is incomplete, but an important facet of this enzyme is that effectors of maximal activity differ between tissues.

For example, in rat kidney, metabolic acidosis is accompanied by high glutamine extraction from the circulation and increased glutaminase activity. However, in skeletal muscle, liver, lung and adipose tissue, glutaminase activity is not affected by acidosis (Watford 1991). Under immunological challenge, glutaminase activity increases in lymphoid tissues (Ardawi and Newsholme 1982), but decreases in rat skeletal muscle (Ardawi and Majzoub 1991c). With glucocorticoid treatment, glutaminase activity is increased in intestinal mucosal cells and unchanged in skeletal muscle. The particular function of glutamine in specific tissues may therefore correlate with differing effectors of glutaminase activity *in vivo*. Furthermore, tissue-specific regulation of glutaminase activity may help to maintain whole-body glutamine homeostasis when local utilisation of this amino acid is transiently increased.

A number of organs and tissues possess both glutaminase and glutamine synthetase activity which, in the rat, include muscle (King *et al.* 1983), liver (Janicki and Goldstein 1969), kidney (Damian and Pitts 1970), lung (Ardawi 1991a) mucosal cells lining the gut (Sarantos *et al.* 1994; Ardawi and Newsholme 1988b) and adipose tissue (Kowalski and Watford 1994b). The net glutamine balance across these tissues and organs is determined by the difference between the activity of these two enzymes, which in turn may affect their capacity to release glutamine to supply other utilising tissues, such as the intestine and cells of the immune system.

In view of the central role glutamine plays in many metabolic pathways, glutaminase and glutamine synthetase activities represent key steps in the regulation of the supply of glutamine to utilising tissues and organs.

1.8. Glutaminase and glutamine synthetase activity in adipose tissue

Studies performed *in vitro* have demonstrated that rat adipose tissue has relatively high levels of glutaminase activity compared to that of other tissues such as kidney and spleen (Kowalchuk *et al.* 1988). Kowalchuk *et al.* also reported differences in the maximal activity between depots with a lower activity in epididymal depot and interscapular brown adipose tissue than in lumbar and interscapular white adipose tissue. Other studies have

shown that rat adipose tissue also possesses considerable glutamine synthetase activity (Miller 1975; Hackenberg and Miller 1976; Kowalski and Watford 1994b) which is reported to be several fold greater than that of skeletal muscle when expressed per mg protein (Miller 1975; Opara 1993). It is, at present, unclear what mechanisms regulate these enzymes in adipose tissue and such information may help to elucidate this tissue's role in whole-body glutamine metabolism.

In contrast to other tissues which contribute to glutamine metabolism such as muscle, the percentage of protein in rodent adipocytes, dependent on the age and species, is less than 1% of the total cell mass, with 70 to 90% being composed of lipid and most of the remainder being water (Di Girolamo and Mendlinger 1971). Therefore, although the overwhelming interest in adipocyte metabolism relates to the storage and release of lipids, it is of great interest that a relatively small proportion of the cell has the capacity to contribute to amino acid storage and release.

1.9. Guinea-pigs as an experimental model for measurement of glutamine metabolism

Studies of site-specific properties of adipose tissue involve comparing different samples from the same individuals which is relatively easy in laboratory animals. In humans, the inaccessibility of many of the sites precludes this approach and data of site-specific properties are reduced to subcutaneous and sometimes one or two intra-abdominal sites, which are exposed during surgery. Guinea-pigs provide a good experimental model with which to study site-specific properties of adipose tissue essentially because of their size and the fact that all of the main adipose tissue depots described in mammals contain sufficient tissue for analysis (Pond and Mattacks 1985). The availability of sufficient adipose tissue in each depot for several complementary analyses allows site-specific studies of reciprocal or matching processes such as those described in Chapters 2 and 4. Furthermore, the basic organisation of adipose tissue in guinea-pigs is similar to that of humans (Pond *et al.* 1993), with the main exception of the intra-abdominal omental and superficial abdominal depots, which are often very large in humans, particularly older

males (Björntorp 1988). It should also be noted that guinea-pigs are grazers whose natural diet is both low in fat and protein. Therefore, nitrogen conservation and lipogenesis from the amino acid carbons are likely to occur, more so than in more omnivorous species such as rats and humans.

In the *ad libitum*-fed adult guinea-pig, total adipose tissue mass represents approximately 15% of total body mass (Figure 1.4) and is distributed into about ten discrete depots (Figure 1.5). Approximately 50% of the total adipose tissue mass is within the abdominal cavity. These depots include: the greater omentum which is attached to stomach and the spleen, and contains lymphatic tissue and numerous blood vessels including branches of the splenic artery, and is drained by the hepatic portal vein. The mesentery is connected to the small and large intestine and contains numerous lymph nodes; its blood supply also drains into the hepatic portal vein. The perirenal depot surrounds the kidneys and extends along and dorsal wall of the abdomen. The epididymal depot is situated above the epididymes, which are attached to the testes (in guinea-pigs, both are devoid of lymph nodes). The epididymal depot is often large in rodents but minimal in humans. The superficial sites include; in front of and behind fore-arm, interscapular hump, and inguinal which extends around the side and ventral groin and has a limited blood supply. The intermuscular sites include: cervical which is located under the musculus trapezius of the neck and the popliteal depot which is enclosed by skeletal muscle of the hind limb, bound laterally by the biceps femori, medially by the semitendinosus and semimembranosus and ventrally by the gastrocnemius muscles. Both of these intermuscular depots contain large lymph nodes and share blood supplies with skeletal muscle.

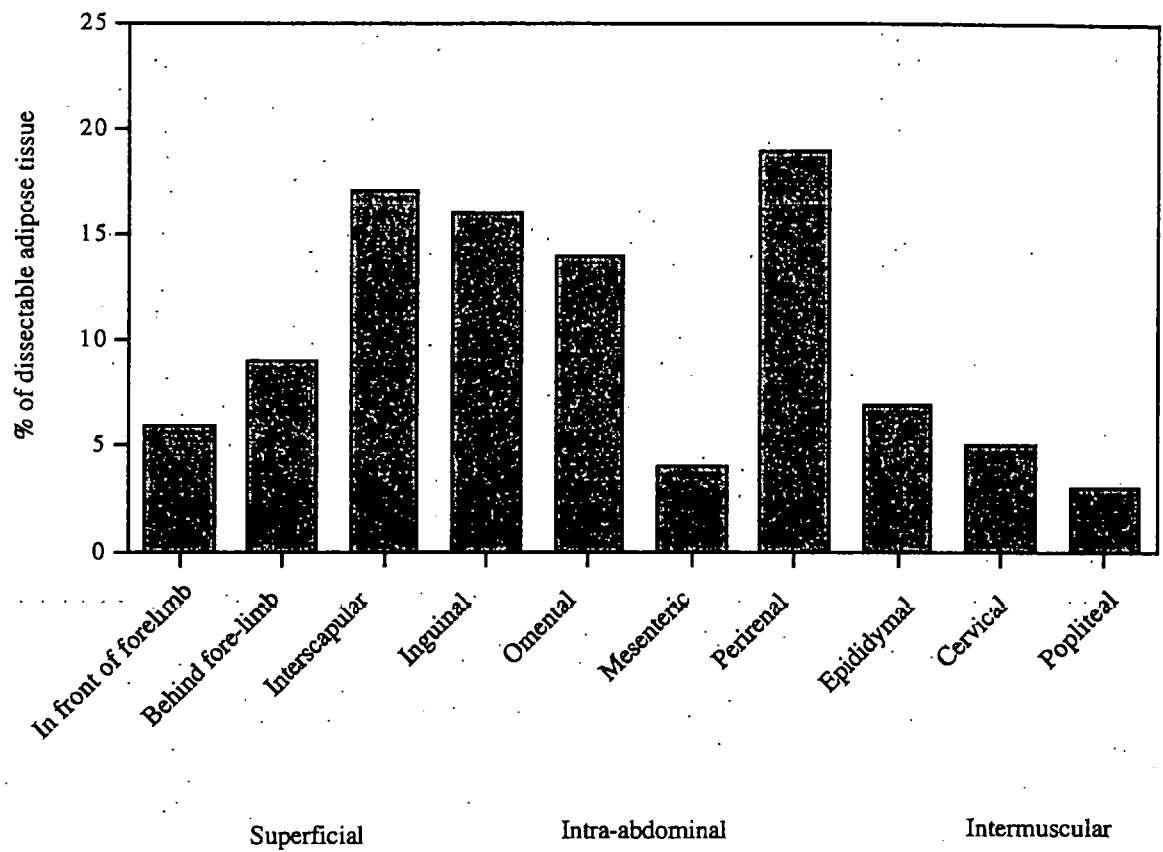


Figure 1.4 Graph showing relative percentage mass of total dissectable adipose tissue in the adult male guinea-pig (data for this figure taken from Pond *et al.* 1992).

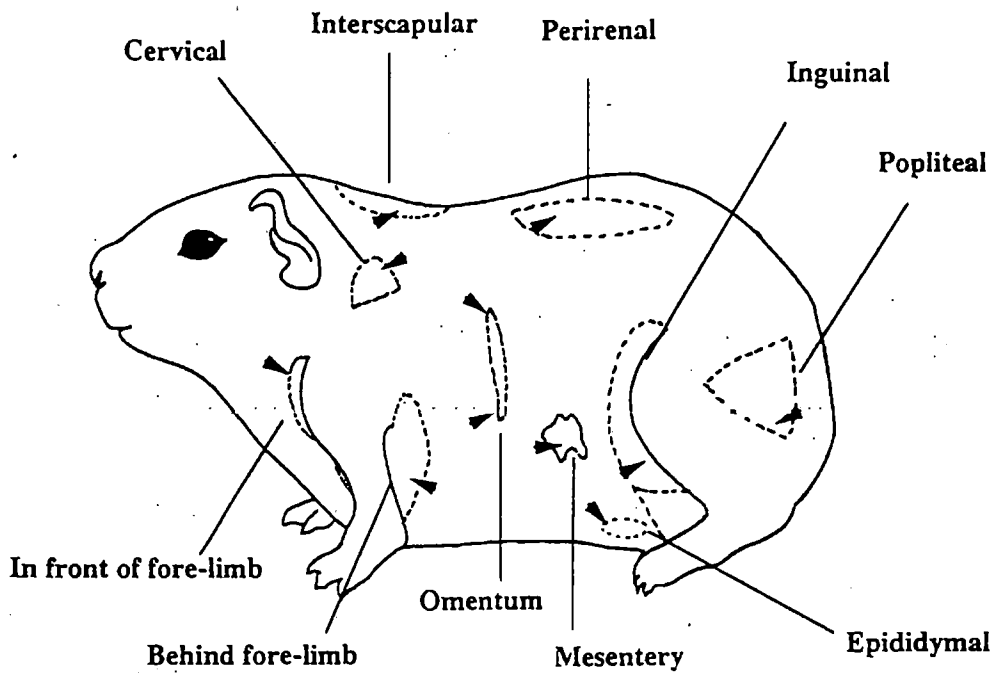


Figure 1.5 Approximate anatomical location of the major adipose tissue depots in the adult guinea-pig. Arrows represent approximate site that samples of adipose tissue were taken ^{from} for the studies of glutamine metabolism described in this thesis.

1.10 Hypothesis

Mammalian adipose tissue utilises and produces glutamine, however, its exact role in the metabolism of this amino acid in the body is not clear.

Hypothesis:

1. As with the metabolism of lipids, there are site-specific differences in the capacity of adipose tissue to utilise and produce glutamine, and in its regulation.
2. The capacity of adipose tissue to utilise and produce glutamine alters in a site-specific manner with different physiological conditions.
3. Adipose tissue contributes significantly to the regulation of whole-body glutamine supply.

1.11 Aims

1. To measure the effect over a time course of fasting, feeding and exercise on the key enzymes involved in glutamine utilisation and production, namely, glutaminase and glutamine synthetase, in extracts of adipose tissue from different adipose sites of the guinea-pig.
2. To measure the effect of different hormones on glutamine uptake and release *in vitro* into and from isolated adipocytes from different adipose sites of the guinea-pig.
3. To determine whether there are site-specific differences in the activity of glutaminase and glutamine synthetase in adipose tissue and correlate these data with the data for glutamine uptake and release into and from isolated adipocytes prepared from different adipose sites.

Chapter 2

**A study of glutaminase activity
following fasting, feeding and
exercise in adipose tissue from
different depots**

2.1. Introduction

The regulation of glutamine utilisation in adipose tissue may involve changes in transmembrane fluxes that alter the uptake and intracellular availability, together with activity of enzymes that metabolise this amino acid. Isolated adipocytes can utilise significant quantities of glutamine, and relatively high levels of glutaminase have been measured in adipose tissue extracts prepared from different depots of the rat (Kowalchuk *et al.* 1988). Furthermore, specific cDNA probes have revealed high levels of kidney-type glutaminase mRNA in adipose tissue (Watford 1991). The physiological significance of adipose tissue's capacity to hydrolyse glutamine is as yet unclear. An understanding of the effect of different physiological conditions and associated hormonal changes on glutamine uptake (discussed in Chapter 3) and metabolism in adipose tissue may therefore help to evaluate this tissue's contribution to whole-body glutamine homeostasis. The mitochondrial enzyme glutaminase catalyses the stoichiometric formation of ammonium and glutamate from hydrolysis of the amide group of glutamine in the reaction shown in Figure 2.1.

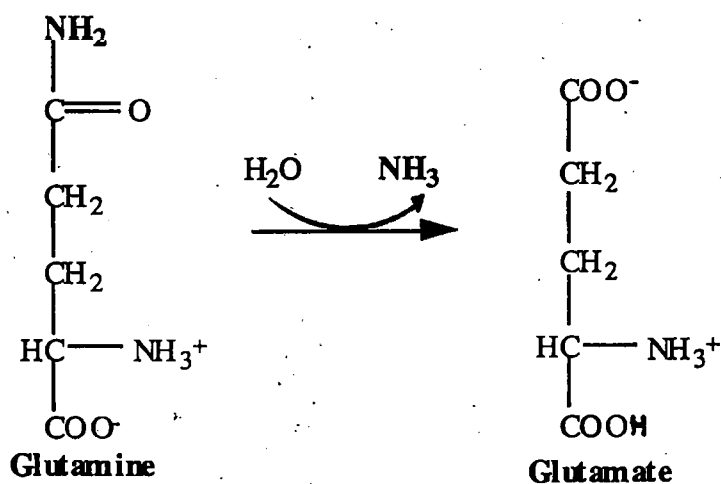


Figure 2.1 Reaction catalysed by glutaminase.

Site-specific differences in glutaminase activity measured in adipose tissue homogenates have previously been reported (Kowalski and Watford 1994b) suggesting that some adipose sites may have a greater capacity than others to hydrolyse glutamine. In the present study the maximal activity of glutaminase was measured in homogenates of adipose tissue from all of the major depots in the guinea pig.

In addition, a preliminary study on the effects of feeding a saturated fat-enriched diet on glutaminase activity in adipose tissue extracts was carried out as previous workers have shown that long chain fatty acyl-CoA derivatives at high concentrations were effective inhibitors of purified pig kidney and brain glutaminase (Kvamme and Torgner 1974, Kvamme and Torgner 1975). A similar result measuring glutaminase activity in rat kidney cortex tubules was also reported by (Baverel *et al.* 1984). These studies did not at the time link these findings with the metabolism of glutamine in adipose tissue and represent a link between the metabolism of amino acids and lipids in this tissue.

Determination of physiological factors that affect adipose tissue glutaminase activity from different anatomical sites may further help to elucidate the contribution of adipose tissue to the regulation of local and whole-body glutamine metabolism.

2.1.1. Hypothesis:

There are site-specific differences in the activity of glutaminase in adipose depots that may be altered with different physiological conditions such as feeding, fasting and exercise.

Such alterations in the activity of glutaminase in different adipose tissue depots with changing physiological conditions can be interpreted functionally and may serve to regulate glutamine supply to local tissues and the whole body.

2.1.2. Aims:

The aims of this study were to investigate *ex vivo* the effect of the physiological conditions of feeding, fasting and exercise on glutaminase activity in adipose tissue from different anatomical sites of the guinea-pig.

2.2. Materials and Methods

2.2.1. Chemicals:

Chemicals and enzymes are detailed in Appendix II.

2.2.2. Dissection Procedure

Animals were sacrificed between 0930 and 1030 h with an intra-peritoneal overdose injection of 3% pentobarbital dissolved in phosphate buffered saline.

0.5 g samples of adipose tissue were immediately dissected onto ice from the following sites (shown in Figure 1.5, Chapter 1) :

4 Subcutaneous sites : in front of fore-limb and shoulder, behind fore-limb, interscapular hump and inguinal.

2 Intermuscular sites : cervical (under the medial to trapezius muscle of the neck) and popliteal.

5 intra-abdominal sites : greater omentum surrounding the greater curvature of the stomach (both adjacent to, and far away from the spleen), mesenteric, (the blood supply from these sites drains into the portal system), perirenal, and epididymal.

In each dissection adipose tissue samples were taken from approximately the same location in each site (Figure 1.5, Chapter 1). Samples of adipose tissue (except omental adjacent to spleen), were taken from sites away from visible blood vessels and lymph nodes to ensure that the preparations did not include any lymphatic tissue (which is known to possess glutaminase activity).

2.2.3. Preparation of homogenates

The dissection took up to a maximum of 20 minutes. 0.5 g of tissue from each adipose site was tightly wrapped in foil and stored at -70°C for up to 4 weeks for the subsequent glutaminase measurements (preliminary experiments indicated that there was no significant

difference in maximal enzyme activity between extracts of frozen and freshly prepared tissues).

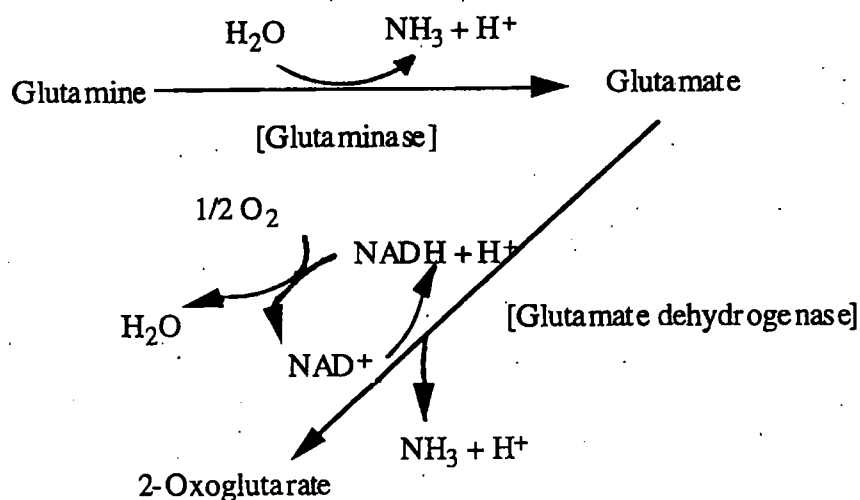
After thawing, the tissue was chopped with scissors and homogenised for 30 seconds (polytron homogeniser, setting 4) in 2.5 ml of ice cold extraction buffer; K_2HPO_4 0.075 M, KH_2PO_4 0.075 M, EDTA (Na^+) 1 mM, Tris aminomethane 0.05 M, pH 8.6. The tissue samples were kept on ice throughout this procedure. The samples were centrifuged for 10 minutes at 400 g using a MSE Coolspin centrifuge. The resultant supernatant constituted the sample for assay of glutaminase, and the pellet was retained for protein determination using a modified method of Bradford (1976) (see Appendix I).

2.2.4. Glutaminase Assay

Glutaminase activity was assayed by a sampling method which measures glutamate formation described by Curthoys & Lowry (1973):

Glutamate is determined by using glutamate dehydrogenase, (GIDH, L-Glutamate: NAD(P) oxidoreductase, deaminating, EC 1.4.1.13) that deaminates it to 2-oxoglutarate coupled to NAD/NADH.

Principle:



NADH formation is measured by the extinction change at 340 nm, and is proportional to the amount of L-glutamate that was present in the sample (Bernt and Bergmeyer 1974).

200 µl aliquots of the adipose tissue homogenates were added in duplicate to 800 µl of assay medium containing phosphate buffer, 150 mM (equimolar mixture K_2HPO_4 , KH_2PO_4), EDTA, 0.02M, Tris aminomethane, 50 mM, Triton X-100, 0.05%, pH 8.6. The reaction was started by the addition of 100 µl of L-glutamine 20 mM, freshly prepared, and the samples were then incubated at 37°C for 20 minutes. Two sets of blanks were assayed in duplicate: 200 µl extraction buffer was added instead of sample homogenate to measure non-enzymatic conversion of glutamine to glutamate, and 100 µl distilled water instead of L-glutamine to measure endogenous glutamate.

The reaction was terminated by the addition of 200 µl cold 25% perchloric acid. Samples were left on ice for 5 minutes, and centrifuged for 2 minutes at 10,000 g in an Eppendorf minifuge. The samples were then neutralised using 10 µl of Universal indicator, 30 µl KOH 40%, and 10 to 20 µl triethanolamine/KOH (0.5 M/ 2 M) and centrifuged again (10000 g for 2 minutes) to remove precipitate formed during neutralisation.

Samples were either assayed immediately for L-glutamate or stored frozen at -20 °C for no more than 4 weeks before being assayed.

2.2. 5. Assay for L-glutamate

200 µl aliquots of centrifuged samples were added to 980 µl of assay medium; glycine 0.5 M, hydrazine hydrate 0.4 M, Tris aminomethane 0.1 M, EDTA 2 mM, ADP 33.5 mM, β-NAD 27 mM, pH 9.0. Absorbance measurements were taken at wavelength 340 nm (Unican 8625 spectrophotometer) and 20 µl glutamate dehydrogenase (ca. 10 mg protein ml⁻¹) added to each cuvette. Samples were incubated at room temperature for 50 minutes and the extinction of NADH was measured at the end of this time by spectrophotometry.

2.2.6. Presentation of data and statistical analysis

Glutaminase activity was expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet tissue and $\text{nmol min}^{-1} \text{mg}^{-1}$ protein in each sample (means of the values of total protein measured in 1 g of adipose tissue from different depots are shown in Appendix I). Statistical analysis was performed on the means of the data for homologous depots of different guinea-pigs using Student's t-test and within treatment differences were analysed by ANOVA.

A post-hoc Tukey test was used to test for statistical significance within treatment groups.

2.3. Experiments

2.3.1 Preliminary study on the effect of fasting and feeding a standard diet or a 15% fat-enriched diet on glutaminase activity in different adipose depots

A preliminary study was carried out to ascertain if fasting and feeding a standard diet or 15% fat-enriched diet had any effect on adipose tissue glutaminase activity *ex vivo*. Animals were either fed a standard diet comprising Regular Guinea-Pig chow (RGP) purchased from SDS Ltd, Waltham, Essex, containing 50% carbohydrate, of which 27% was starch, 3.4% crude lipid, of which 16% were saturated fatty acids and 17% were monoenoics and 17.4% crude protein, approximate BCAA composition; leucine, 12, isoleucine 7.3, valine, 9.0 mg g^{-1} (see Appendix II for full details of the average composition of diet), or fed a 15% fat enriched diet comprising of 85% powdered RGP and 15% lard in which 40% of the triacylglycerols were saturated (see Appendix II for diet preparation).

The guinea pigs used for this study were male Bolivians born and raised at the Open University. They were aged between 12 and 13 months (mean 12.4 ± 0.5 months), body mass, 1021 to 1402 g (mean 1158 ± 117 g). Twelve animals were randomly assigned to three treatment groups:

- (i) Fasted (chow removed 18 hours before sacrifice).
- (ii) standard diet, sacrificed 30 minutes after the end of a meal following an overnight fast.
- (iii) 15% fat enriched diet, sacrificed after the end of a meal following an overnight fast.

Animals in groups (i) and (ii) were given diet of RGP with added vitamins, standard pellet size 4 mm, manufactured by Labsure. Animals in group (iii) were fed a 15% w/w fat-enriched experimental diet, over a period of at least 4 weeks. All three treatment groups were given unrestricted access to water with added vitamin C (0.1 mg ml⁻¹). They were also given carrot, cabbage and apple 5 days per week, and hay each morning which was consumed by 1600 h.

To ensure that the animals consumed a significant quantity of food on the day of the experiment and adjusted to their new environment, feeding regime and diet, they were transferred to single cages (floor area 0.45 m²), 8 weeks prior to the experiment. In groups (ii) and (iii) chow was removed for 18 hours overnight consecutively for 6 weeks prior to the experiment, they were therefore trained to expect food in the morning. Body weight was recorded weekly over the 8 week period.

On the day of the experiment the guinea-pigs were given food as normal at 0900 h which was removed after 30 minutes. The animals were sacrificed 30 minutes after food removal. The initial time course of 30 minutes after the feeding period was chosen as some of dietary absorbed amino acids, specifically BCAAs, would be expected to be present in the circulation at this time (Elia *et al.* 1989).

Samples of adipose tissue were taken from sites described previously (Section 2.2.3) with a reference sample from the kidney, glutaminase activity was determined as described in section 2.2.3. All samples were assayed as a batch and enough tissue was dissected to enable both glutaminase and glutamine synthetase to be measured from all depots of the same animals.

2.3.2 The effect of fasting, feeding (standard diet) and exercise on glutaminase activity in extracts of adipose tissue from different sites over a time course of 240 minutes

The guinea pigs used in this study were male Bolivians born and raised at the Open University. However, the animals used in this study were aged 6 months old, compared to those used in the previous study which were between 12 and 13 months old (Section

2.3.1). The mean body mass of the guinea-pigs (962 ± 31 g) was also less than that of the animals in the previous study.

Guinea-pigs were transferred to individual cages (floor area 0.45 m^2) 2 to 3 weeks prior to the experiment to enable them to adjust to their new environment and feeding regime. Animals were randomly assigned to three experimental groups; (i) fasted (chow removed 18 hours before sacrifice), fed (overnight fasted, fed at 0900 then sacrificed immediately after the 30 minute feeding period, or at 30, 60 and 240 minutes after the end of the feeding period, (iii) exercised (fasted overnight, exercised for 10 minutes in a 180 cm^2 pen then sacrificed immediately or at 30, 60 and 240 minutes after exercise). The animals in the time-course groups were kept in a quiet environment until sacrificed so as not to induce additional excitement after exercise or feeding. In the fed groups, the animals consumed a mean of 7.1 ± 0.45 g of chow in the allotted 30 minute feeding period.

Samples of adipose tissue were taken from sites described previously and glutaminase activity was determined as described in Section 2.2.3.

Changes in enzyme activity within and between depots under different physiological conditions were compared with the uptake of glutamine in isolated adipocytes from selected depots incubated with hormones (or analogues) expected to be present in the circulation under the experimental conditions shown above (described in Chapter 3).

2.4. Results

2.4.1. Preliminary study on the effect of fasting and feeding a standard diet or a 15% fat-enriched diet on glutaminase activity in different adipose sites

2.4.2. Animal body mass and feeding behaviour over an 8 week period

After 14 consecutive periods of overnight food restriction, the feeding behaviour of the guinea pigs on the standard diet and on the 15% fat experimental diet was observed. At 0900 h, all of the animals in these groups appeared eager for food and began feeding as soon as it was given to them. The four animals observed all fed continuously for approximately the first 15 minutes, after which feeding was interrupted by short breaks lasting an average of 3.6 minutes. No wastage was observed at the end of 30 minutes. On the day these observations were made, the animals consumed 8.2 ± 0.92 g of food over the monitored time period, regardless of diet type.

The body mass of the guinea pigs used in this study did not change significantly over the 8 week period between, nor did it differ between the 3 treatment groups (Table 2.1).

Experimental group	Fasted (on day of experiment)	Fed standard diet (fasted overnight)	Fed 15 % fat diet (fasted overnight)
<i>Body mass at start of 8 week period</i>	1148.8 \pm 28.4	1134.8 \pm 42.6	1209.3 \pm 58.4
<i>Body mass at end of 8 week period</i>	1158.5 \pm 31.9	1154.75 \pm 48.9	1195.0 \pm 75.9

Table 2.1 Body mass of the guinea-pigs over 8 weeks, values are mean (\pm SE) expressed as grams of body mass, n = 4 per group.

2.4.3 Food consumption

Food intake was measured at the end of a 30 minute feeding period on the day of the experiment to ensure that a known significant quantity had been consumed. There was no significant difference in the quantity of food consumed in the 30 minute feeding period between animals fed on the standard or experimental diet. Animals fed the standard diet consumed a mean of 12.4 ± 0.4 g, and animals fed the experimental diet consumed a mean of 13.0 ± 3.9 g.

2.4.4 Preliminary study of the effect of fasting and feeding either a standard or 15% fat enriched diet on glutaminase activity between different adipose sites

There were large site-specific differences in glutaminase activity when expressed mg^{-1} protein and g^{-1} wet tissue weight, in adipose tissue extracts within the fasted treatment group (Table 2.2). The greatest values of enzyme activity g^{-1} wet tissue weight and mg^{-1} protein were in tissue extracts from the omental (adjacent to spleen) and in the inguinal site (Figure 2.2 and Table 2.2). Mesenteric adipose tissue extracts had the least enzyme activity expressed either as mg^{-1} protein or g^{-1} wet tissue weight (Figure 2.2 and Table 2.2). In the cervical depot, glutaminase activity could not be detected at all. To confirm this result, the glutaminase assay was repeated twice with all samples on consecutive days, but no activity in cervical site could be measured at any time.

After feeding a standard diet, values of glutaminase activity remained significantly higher ($P < 0.05$) in the inguinal and omental (adjacent to spleen) sites than that of all other sites, with the exception of that of the omental (far from spleen) where the large standard errors precluded significance (Figure 2.3). The net effect of feeding (a standard diet) was that glutaminase activity between all sites, except inguinal and omental (adjacent to, and far from, spleen), became more uniform compared with site-specific differences of the fasted values (Table 2.2).

Feeding a 15% fat enriched diet negated the between-site differences in glutaminase activity observed in the inguinal and omental (adjacent to spleen) sites of the standard diet fed animals (Figure 2.4). The least value of glutaminase activity compared to any of the other sites was observed in the intermuscular cervical site (Figure 2.4).

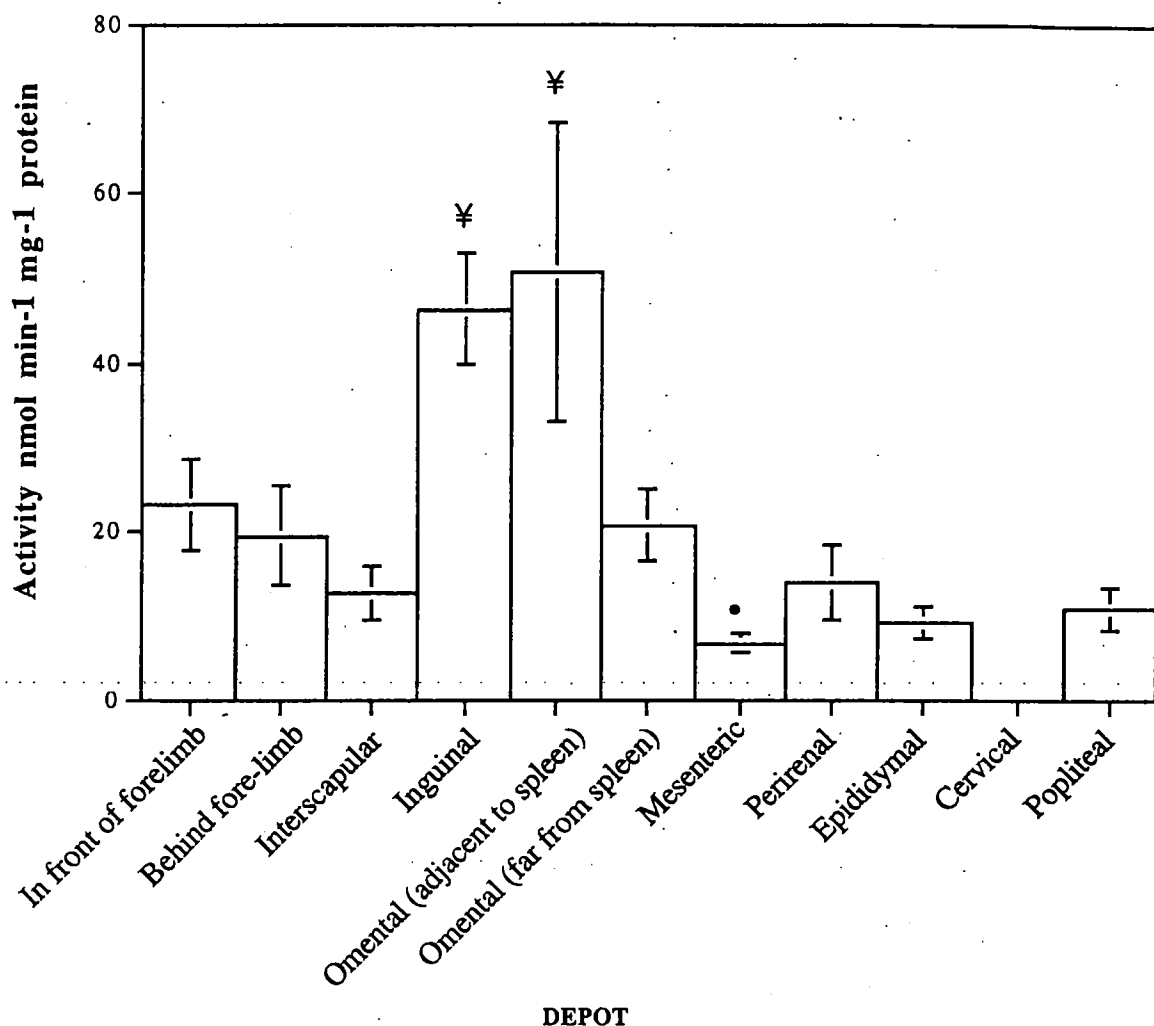


Figure 2.2 Glutaminase activity, in different adipose sites of 12 month old fasted guinea-pigs, nmol substrate utilised min⁻¹ mg⁻¹. ¥ denotes glutaminase activity significantly higher (P<0.05 or less) than that of most of the other sites, • denotes glutaminase activity significantly lower P<0.05 or less) than that of most of the other sites.

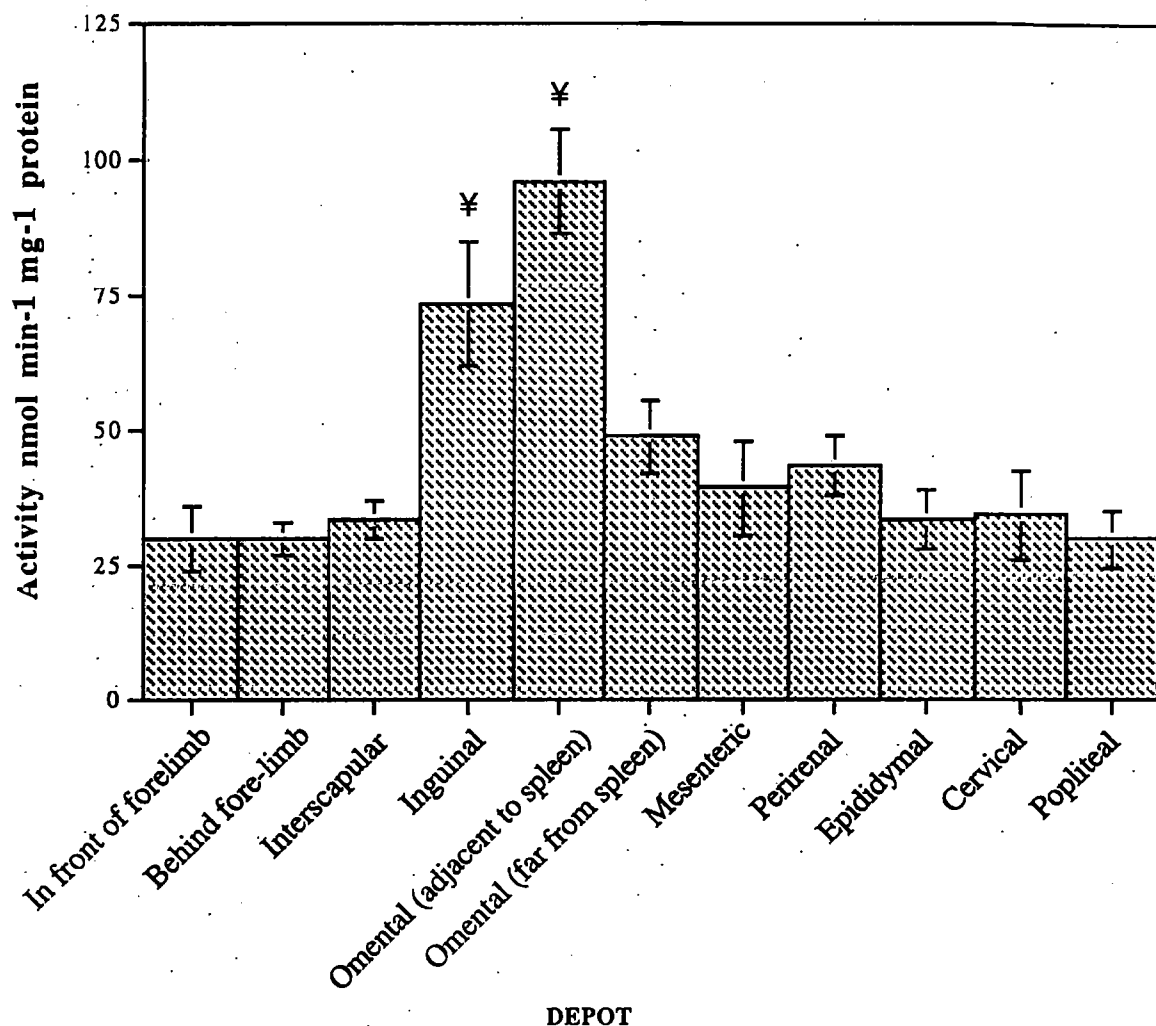


Figure 2.3 Glutaminase activity, in different adipose sites of 12 month old fed guinea-pigs (standard diet), nmol substrate utilised min⁻¹ mg⁻¹. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than that of most of the other sites.

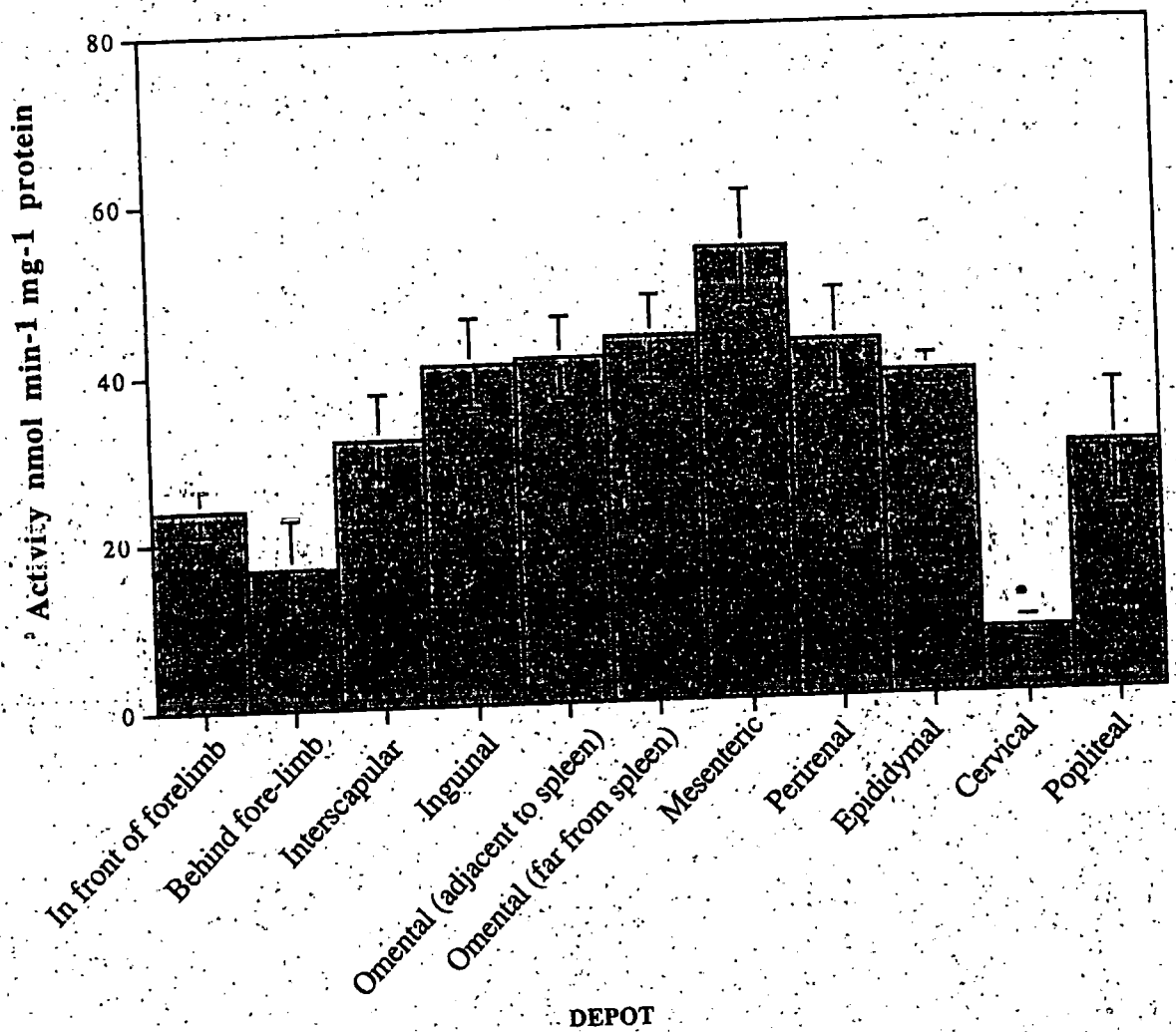


Figure 2.4 Glutaminase activity, in different adipose sites of 12 month old fed guinea-pigs (15% fat enriched diet), nmol substrate utilised min⁻¹ mg⁻¹. • denotes glutaminase activity significantly lower ($P < 0.05$ or less) than most of the other sites.

2.4.5 Site specific differences in the effect of feeding a standard diet or a 15% fat enriched diet on glutaminase activity in different adipose sites

The data in Table 2.2 show that there was little change in glutaminase activity in the in front of fore-limb and the behind fore-limb sites after feeding either a standard diet or a 15% fat enriched diet. Glutaminase activity ($\text{nmol min}^{-1} \text{g}^{-1}$ wet weight and $\text{nmol min}^{-1} \text{mg}^{-1}$ protein) measured in extracts of adipose tissue from interscapular, inguinal, omental (adjacent to and far from spleen), mesenteric, perirenal, epididymal, cervical and popliteal sites in the standard diet fed group increased significantly when measured 30 minutes after the feeding period compared with samples from the fasted group (Table 2.2). Enzyme activity was also significantly increased (g^{-1} wet weight and mg^{-1} protein), in extracts of adipose tissue from the animals fed a 15% fat enriched diet in the interscapular, omental (far from spleen), mesenteric, perirenal, epididymal and popliteal sites compared with fasted animals (Table 2.2).

Glutaminase activity was significantly lower g^{-1} wet weight and mg^{-1} protein, in extracts of adipose tissue from behind fore-arm, inguinal, omental (adjacent to spleen) and cervical sites in the animals fed a 15% fat enriched diet when compared with that of animals fed a standard diet (Table 2.2).

The mean reference values of glutaminase activity obtained from guinea-pig kidney tissue extracts was significantly higher than those measured in adipose tissue extracts when expressed g^{-1} wet tissue weight however, this difference was negated when enzyme activity was expressed mg^{-1} protein (Table 2.2). To the author's knowledge, there have been no reported measurements of glutaminase in guinea-pig kidney, however, the values of enzyme activity mg^{-1} protein shown in the present study are lower than those reported for rat kidney (Watford 1991).

Table 2.2 Effects of fasting and feeding a standard diet or a 15% fat enriched diet on glutaminase activity in extracts of guinea-pig adipose tissue from different sites 30 minutes after a meal. Values are mean (\pm SEM) expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet weight, and in parentheses, nmol $\text{min}^{-1} \text{mg}^{-1}$ protein, n = 4 per group, n.d. = enzyme activity not detectable.

The statistical differences between the fasted and fed (standard diet) means are denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and between standard diet and 15% fat diet † $P < 0.05$, †† $P < 0.01$. Glutaminase activity significantly greater ($P < 0.05$ or less) than that of most or all other the sites measured from the same animals is denoted by ¥ and • significantly less ($P < 0.05$) than any of the other sites from the same animals.

Depot	Treatment Group		
	Fasted	Fed - standard diet	Fed - 15% fat-enriched diet
<i>Superficial</i>			
In front of fore-limb	160.4 \pm 37.8 (23.2 \pm 5.4)	206.1 \pm 39.8 (29.8 \pm 5.8)	164.2 \pm 18.6 (23.7 \pm 2.7)
Behind fore-limb	136.6 \pm 41.7 (19.5 \pm 5.9)	207.9 \pm 21.6 (29.7 \pm 3.1)	117.3 \pm 31.2 (16.7 \pm 5.9)
Interscapular	82.8 \pm 20.2 (12.31)	216.0 \pm 22.9** (33.2 \pm 3.5**)	208.3 \pm 34.7* (32.1 \pm 5.3*)
Inguinal	234.9 \pm 29.0¥ (46.4 \pm 6.4¥)	381.8 \pm 19.4**¥ (73.4 \pm 11.4**¥)	211.4 \pm 27.9† (40.6 \pm 5.3†)

(Continued on next page).

(Table 2.2 continued).

<i>Intra-abdominal</i>	Fasted	Fed - standard diet	Fed - 15% fat- enriched diet
Omental (adjacent to spleen)	1239.6±585.9 [‡] (39.1±11.6)	3033.6±299.3 [‡] (95.7±9.4 [‡])	1303.8±154.4 ^{††} (41.1±4.8 ^{††})
Omental (far from spleen)	178.7±36.9 (20.7±4.2)	419.2±57.6 ^{**} (48.7±6.7 ^{**})	372.6±43.2 [*] (43.3±5.0 [*])
Mesenteric	35.0±13.4 [*] (6.7±1.2)	271.5±60.1 ^{**} (39.3±8.7 ^{**})	370.1±46.5 ^{***} (53.6±6.7 ^{**})
Perirenal	69.4±33.6 (14.0±4.5)	285.7±34.7 ^{**} (43.2±5.4 ^{**})	279.6±41.6 [*] (42.3±6.3 [*])
Epididymal	74.6±16.1 (9.1±1.9)	272.2±46.2 ^{**} (33.1±5.5 ^{**})	317.4±14.3 ^{***} (38.7±1.8 ^{***})
<i>Intermuscular</i>			
Cervical	n.d.	302.1±72.2 ^{**} (34.3±8.2 ^{**})	60.2±10.5 ^{**††} (7.5±1.3 ^{**††})
Popliteal	79.5±18.9 (10.7±2.5)	220.0±34.0 ^{**} (29.7±5.1 ^{**})	219.3±55.1 [*] (29.6±7.5 [*])
<i>Reference tissue</i>			
Kidney	4820.0±474 (35.1±2.04)	5120±120 (37.4±1.1)	4773±221 (34.8±1.8)

2.4.6. Between-site differences in glutaminase activity with fasting and feeding a standard diet over a time course of 240 minutes

Glutaminase measurements in extracts of adipose tissue from the fasted group gave comparable results to the previous study in that the pattern of between-site differences were similar (Section 2.4.4), although the absolute values of enzyme activity g^{-1} wet tissue and mg^{-1} were lower in this study than in the previous experiments. Differences in enzyme activity between the two studies were not due to the efficiency of the assay as standard concentrations of glutamine were used as a control for inter-assay variation. The lower values of glutaminase activity may in part be due to the difference in ages of the animals used in this study compared to the age of the animals in the previous study (an average of 6 months old for this study and 12 months old for the previous study). In addition, animals in this study consumed less food in the allotted time period than those of the previous experiment (7.4 g in the present study compared to 12.4 g in the previous study).

In the fasted state, glutaminase activity was greatest in extracts of adipose tissue from the intra-abdominal omental (adjacent to the spleen) site compared to that any of the other sites when expressed as $\text{nmol min}^{-1} \text{g}^{-1}$ wet tissue weight (Figure 2.5 and Table 2.3b). The lowest values of enzyme activity, $\text{nmol min}^{-1} \text{g}^{-1}$ wet tissue weight and $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, with fasting was observed in adipose tissue extracts from the portally drained mesenteric site, and could not be detected in adipose tissue from the intermuscular cervical site (Figure 2.5, Tables 2.3b and 2.3c).

Directly after the feeding period, the highest value of glutaminase activity compared to any of the other sites, $\text{nmol min}^{-1} \text{g}^{-1}$ wet tissue weight or $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, was measured in the omental (adjacent to spleen) site (Figure 2.6 and Table 2.3b). The least values of enzyme activity were measured in the in front of fore-limb, interscapular, perirenal and epididymal sites (Figure 2.6, Table 2.3a and b).

At 30 minutes after the feeding period, the highest value of glutaminase activity, compared to any of the other sites, was measured in the inguinal site and values of enzyme activity were not significantly different from one another in any of the remaining sites

(Table 2.3a, b and c, Figure 2.7). Values of glutaminase activity were not significantly different from one another at 240 minutes after feeding (Table 2.3a, b and c, Figure 2.8).

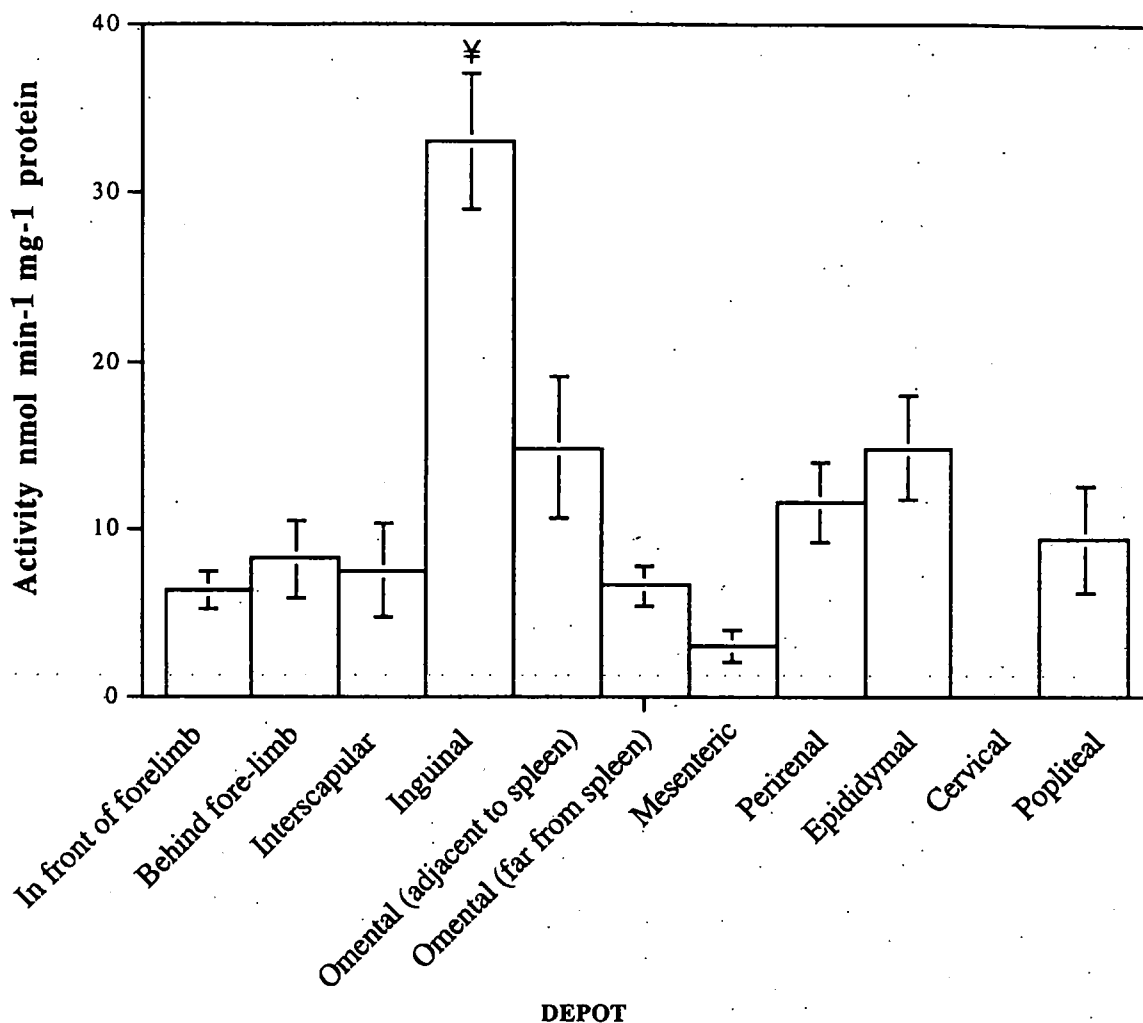


Figure 2.5 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein after an overnight fast of 18 hours. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites.

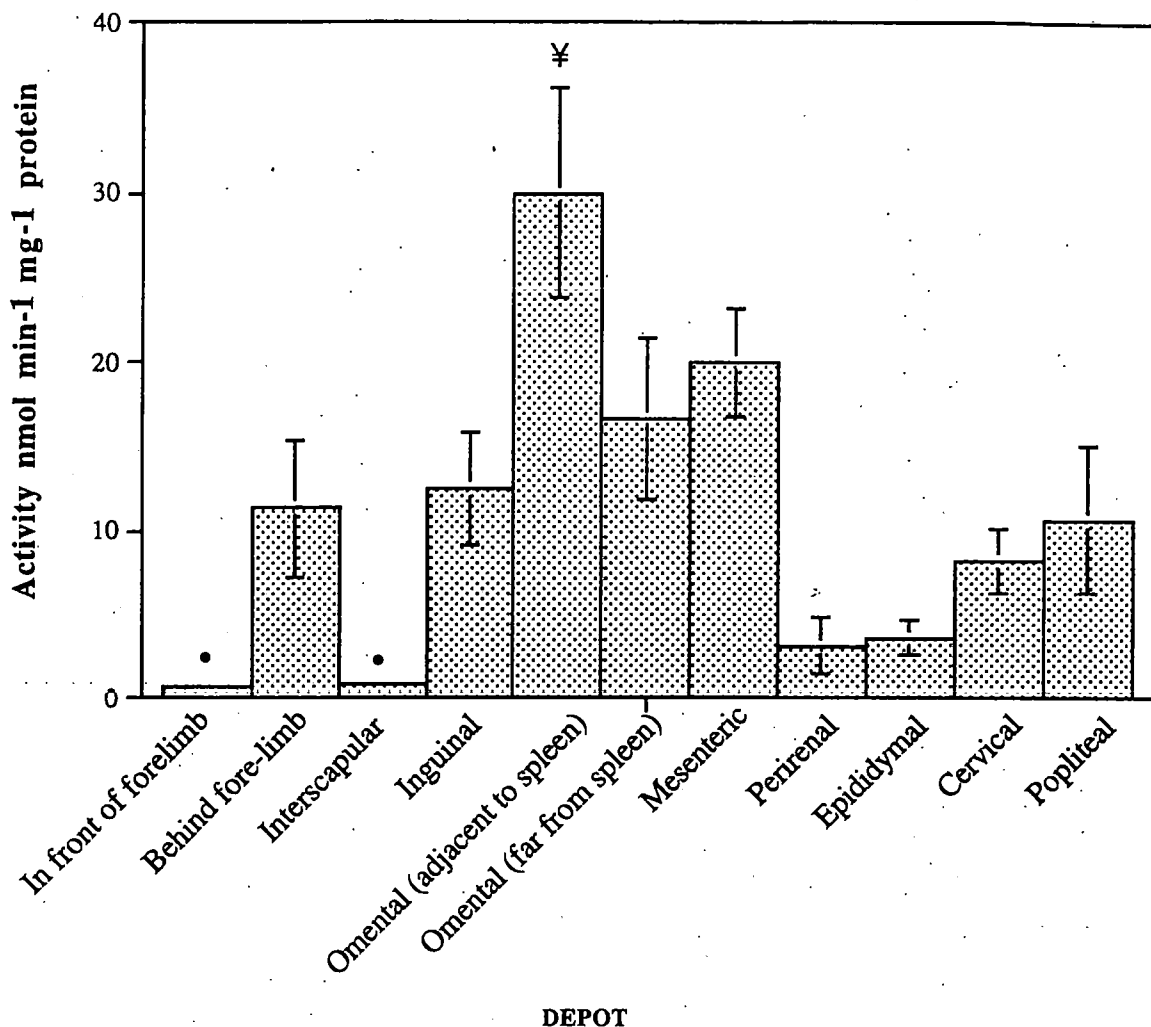


Figure 2.6 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein directly after the feeding period. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites, • denotes significantly lower ($P < 0.05$) than all other sites.

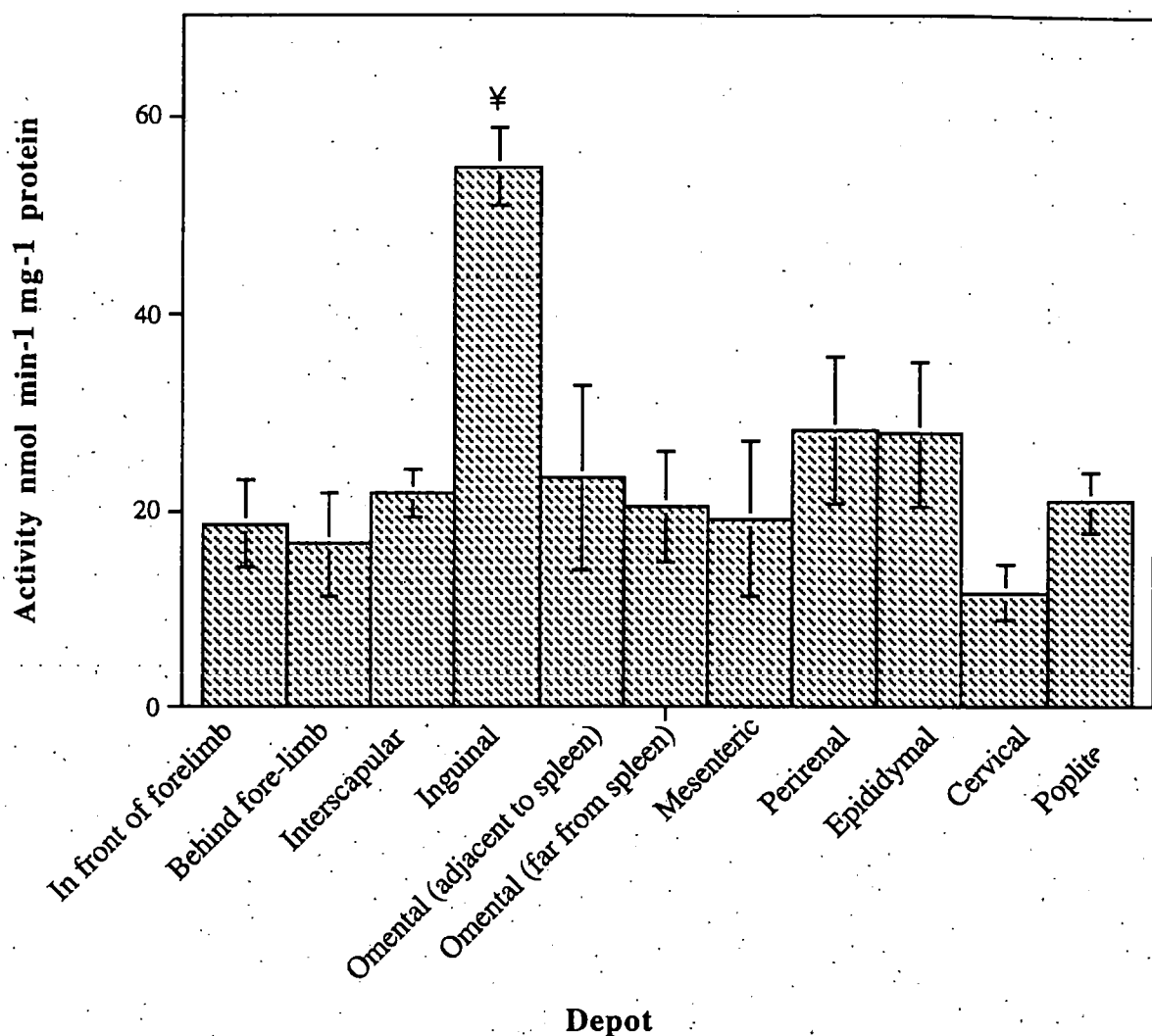


Figure 2.7 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein at 30 minutes after the feeding period. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites. Note different scale from Figs. 2.4 and 2.5.

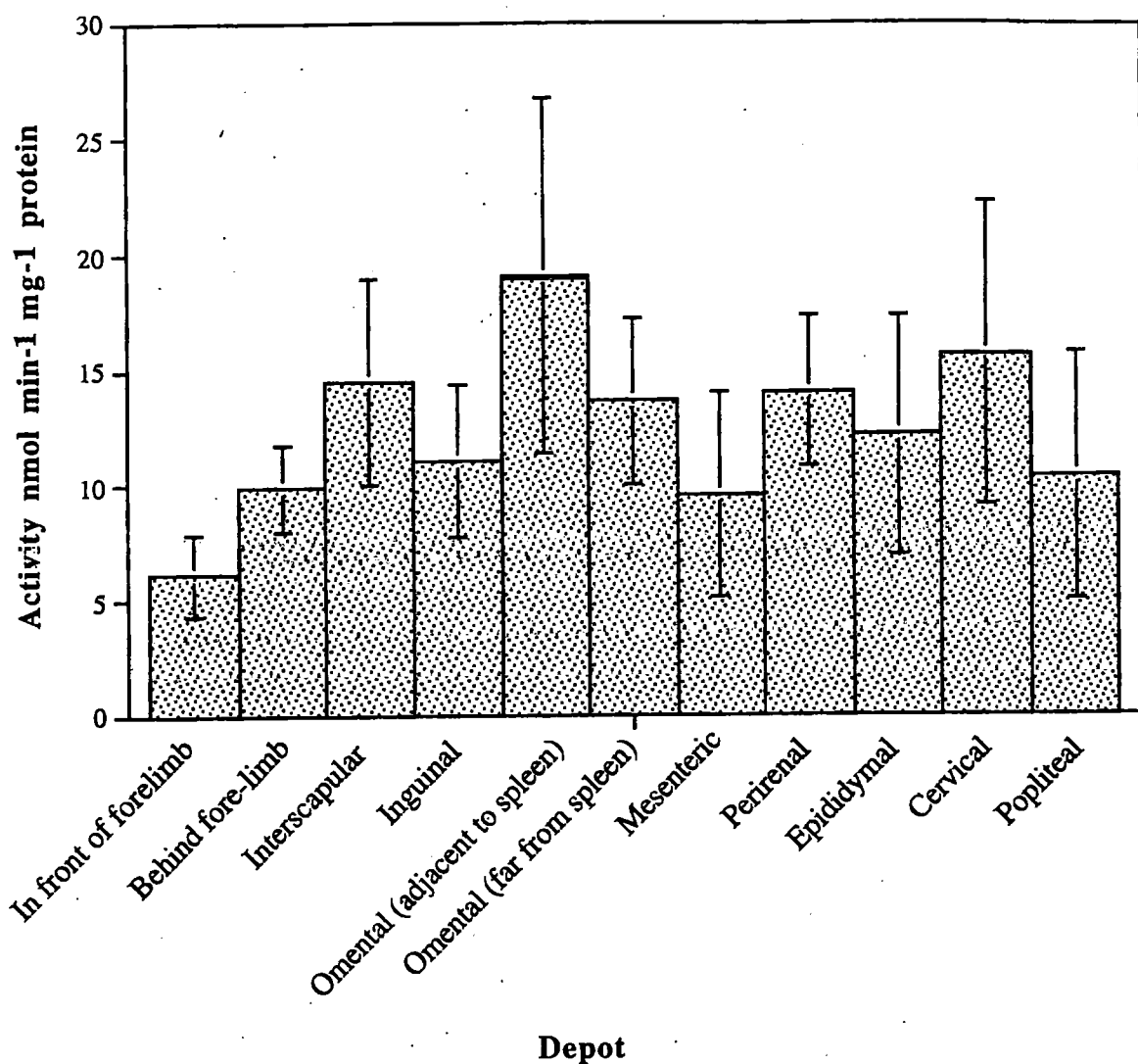


Figure 2.8 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein at 240 minutes after the feeding period. No significant difference in glutaminase is shown between any of the sites measured.

2.4.7 The effect of feeding on glutaminase activity in different adipose depots over a time course of 240 minutes

There was a significant site-specific increase in glutaminase activity, expressed as $\text{nmol min}^{-1} \text{g}^{-1}$ wet tissue weight and $\text{nmol min}^{-1} \text{mg}^{-1}$ protein, directly after the feeding period in extracts of adipose tissue from the portally drained sites: omental (adjacent to spleen), omental (far from spleen) and mesenteric (Table 2.3b) and in tissue extracts from the intermuscular cervical site (Table 2.3c). Glutaminase activity remained elevated to a similar extent 30 minutes after the feeding period in omental (far from spleen), mesenteric and cervical adipose tissue extracts (Tables 2.3b and 2.3c).

Directly after the feeding period there was a decrease in enzyme activity g^{-1} wet tissue weight and mg^{-1} protein, in adipose tissue extracts from the following sites; in front of fore-limb, interscapular, inguinal, (Table 2.3a) epididymal, perirenal (Table 2.3b). However, by 30 minutes after the feeding period glutaminase activity was significantly higher, when expressed as either g^{-1} wet tissue weight or mg^{-1} protein, compared with samples from the fasted group, in tissue from in front of fore-limb, behind fore-limb, interscapular, inguinal, and popliteal sites (Tables 2.3a and 2.3c). Increases in enzyme activity ranged between 1.6 to 3.5-fold in adipose tissue from the superficial sites, 3 and 5-fold in mesenteric and omental (far from spleen) respectively, and 2 fold in popliteal.

At 60 minutes after feeding, no measurable glutaminase activity could be detected in any of the sites studied apart from omental (adjacent to spleen) where the activities g^{-1} wet tissue weight and mg^{-1} protein, were significantly higher than the fasting state (Table 2.3b).

At 240 minutes after feeding, there was no significant difference in enzyme activity, g^{-1} wet tissue weight and mg^{-1} protein, compared with corresponding values from the fasted group in the following sites: in front of forelimb, behind forelimb, interscapular omental (adjacent to spleen), mesenteric, perirenal, epididymal, and popliteal. In adipose tissue extracts from the inguinal site, enzyme activity, g^{-1} wet tissue weight and mg^{-1} protein, was significantly lower than those of the corresponding mean

fasted values but similar to that measured directly after the feeding period (Table 2.3a). In adipose tissue from omental (far from spleen) and cervical sites, glutaminase activity was increased compared with that of the fasted values measured in these sites (Table 2.3b and 2.3c).

2.4.8. Glutaminase activity in adipose tissue from the greater omentum (adjacent to spleen)

As with the previous study (Section 2.4.4), glutaminase activity, g^{-1} wet tissue weight, measured in samples taken from the greater omentum (adjacent to spleen), was much higher than any of the other sites measured in this study (Table 2.3b). However, when enzyme activity was expressed mg^{-1} protein, values were comparable with those measured in other adipose sites. The reduced values of enzyme activity, when expressed per mg protein, were due to the high protein content of tissue taken from the omentum close to spleen ($29.6 \pm 3.2 \text{ mg g}^{-1}$ whole adipose tissue, Table 2.3b) compared with omentum far from spleen ($8.5 \pm 0.5 \text{ mg g}^{-1}$ whole adipose tissue, Table 2.3b) and other depots. Therefore, when expressed g^{-1} wet tissue weight, omental (adjacent to spleen) appears to have a much greater capacity, indicated by these measurements of glutaminase activity, to utilise glutamine compared with all other adipose sites measured. In addition, glutaminase activity, expressed mg^{-1} protein, measured in omental adipose tissue (adjacent to spleen) was still higher compared to that of some of the other sites and compared to all other sites directly after the feeding period (Table 2.3b and Figure 2.6).

Table 2.3a Effect of feeding over a time course of 240 minutes on glutaminase activity in extracts of superficial adipose tissue

Values are mean (\pm SEM) expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet weight, in parentheses nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein.

Fasted $n = 6$, fed + 240 minutes $n = 5$, remaining groups $n = 4$, n.d. = enzyme activity not detectable The statistical differences between the fasted and fed means are denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Significantly higher ($P < 0.05$ or less) from most or all other sites measured in the same condition studied is denoted by ¥. Significantly lower ($P < 0.05$ or less) from most or all other sites measured in the same condition studied is denoted by •.

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
In front fore-limb	41.0 \pm 8.2 (6.4 \pm 1.1)	5.0 \pm 2.0** (0.7 \pm 0.2**•)	134.5 \pm 31.3** (18.7 \pm 4.4*)	n.d.	44.0 \pm 13.1 (6.1 \pm 1.8)
Behind fore-limb	56.1 \pm 13.4 (8.2 \pm 2.3)	70.5 \pm 38.2 (11.3 \pm 4.1)	126.3 \pm 40.1 (16.6 \pm 5.3)	n.d.	75.0 \pm 14.5 (9.9 \pm 1.9)
Interscapular	47.2 \pm 13.4 (7.5 \pm 2.8)	11.4 \pm 6.8* (1.8 \pm 0.4*•)	146.5 \pm 16.1** (21.8 \pm 2.4**)	n.d.	97.0 \pm 30.2 (14.5 \pm 4.5)
Inguinal	142.8 \pm 19.3¥ (33.0 \pm 4.0¥)	91.1 \pm 7.0* (12.5 \pm 3.4***)	256.6 \pm 18.8** (54.9 \pm 4.0**¥)	n.d.	51.7 \pm 15.4** (11.1 \pm 3.3**)

Table 2.3b Effect of feeding over a time course of 240 minutes on glutaminase activity
in extracts of intra-abdominal adipose tissue

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
Omental (adj. to spleen)	439.2±132 ^Y (14.9±4.2)	1000.1±100** (30.0±6.2** ^Y)	436.7±77.7 (23.3±9.4)	2113.6±672* (62.4±28.3*)	640.5±260 (19.1±7.7)
Omental (far from spleen)	47.3±8.4 (6.6±1.2)	145.1±33.5** (16.7±4.8*)	162.1±45.1** (20.5±5.7*)	n.d.	108.0±28.7* (13.7±3.6*)
Mesenteric	30.5±4.3 (3.0±1.0*)	142.5±33.5** (20.0±3.2**)	138.0±56.9* (19.2±7.9*)	n.d.	69.4±32.5 (9.6±4.5)
Perirenal	72.1±12.8 (11.6±2.4)	38.7±10.3** (5.1±1.7*)	182.5±48.2 (28.3±7.5)	n.d.	91.1±21.0 (14.1±3.3)
Epididymal	92.2±19.3 (14.9±3.1)	37.2±5.0** (3.6±1.0***)	160.5±45.9 (27.9±7.4)	n.d.	126.2±15.3 (12.2±5.2)

Table 2.3c Effect of feeding over a time course of 240 minutes on glutaminase activity
in extracts of intermuscular adipose tissue

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
Cervical	n.d.	67.2±12.1 (8.2±1.9)	108.8±24.8 (11.7±2.9)	n.d.	125.7±29.2 (15.7±6.6)
Popliteal	79.5±18.9 (9.4±3.2)	72.0±23.4 (10.6±4.4)	151.3±21.2* (20.9±3.1*)	n.d.	70.2±36.3 (10.4±5.4)

2.4.9 Between-site differences in glutaminase activity with exercise over a time course of 240 minutes

Directly after exercise, glutaminase activity was significantly higher in adipose tissue from the inguinal site ($P < 0.05$) than that of any of the other sites measured (Figure 2.9). The least values of glutaminase activity, at this time point, were observed in omental (far from spleen) and popliteal sites, with no detectable enzyme activity in the perirenal and epididymal sites (Figure 2.9).

At 30 minutes following exercise, the highest value of enzyme activity was measured in the omental (adjacent to spleen) site, which was significantly higher than that measured in any of the other sites with the exception of perirenal. The lowest value compared to that of most other sites was measured in the behind fore-limb site (Figure 2.10). When measured at 60 minutes after exercise there were no significant differences in glutaminase activity between sites, with the exception that enzyme activity in the omental (adjacent to spleen site) was higher than that of the perirenal and popliteal sites (Figure 2.11).

When measured at 240 minutes after exercise, between-site differences in glutaminase activity were similar to those shown in the values for adipose tissue from sedentary fasted group; the highest value of enzyme activity was measured in the inguinal sample and the lowest value was measured in the mesenteric sample (Figure 2.12). However, at this time point, glutaminase activity was detectable in adipose tissue from the cervical site (Figure 2.12) whereas it could not be detected in the corresponding tissue from the fasted sedentary group.

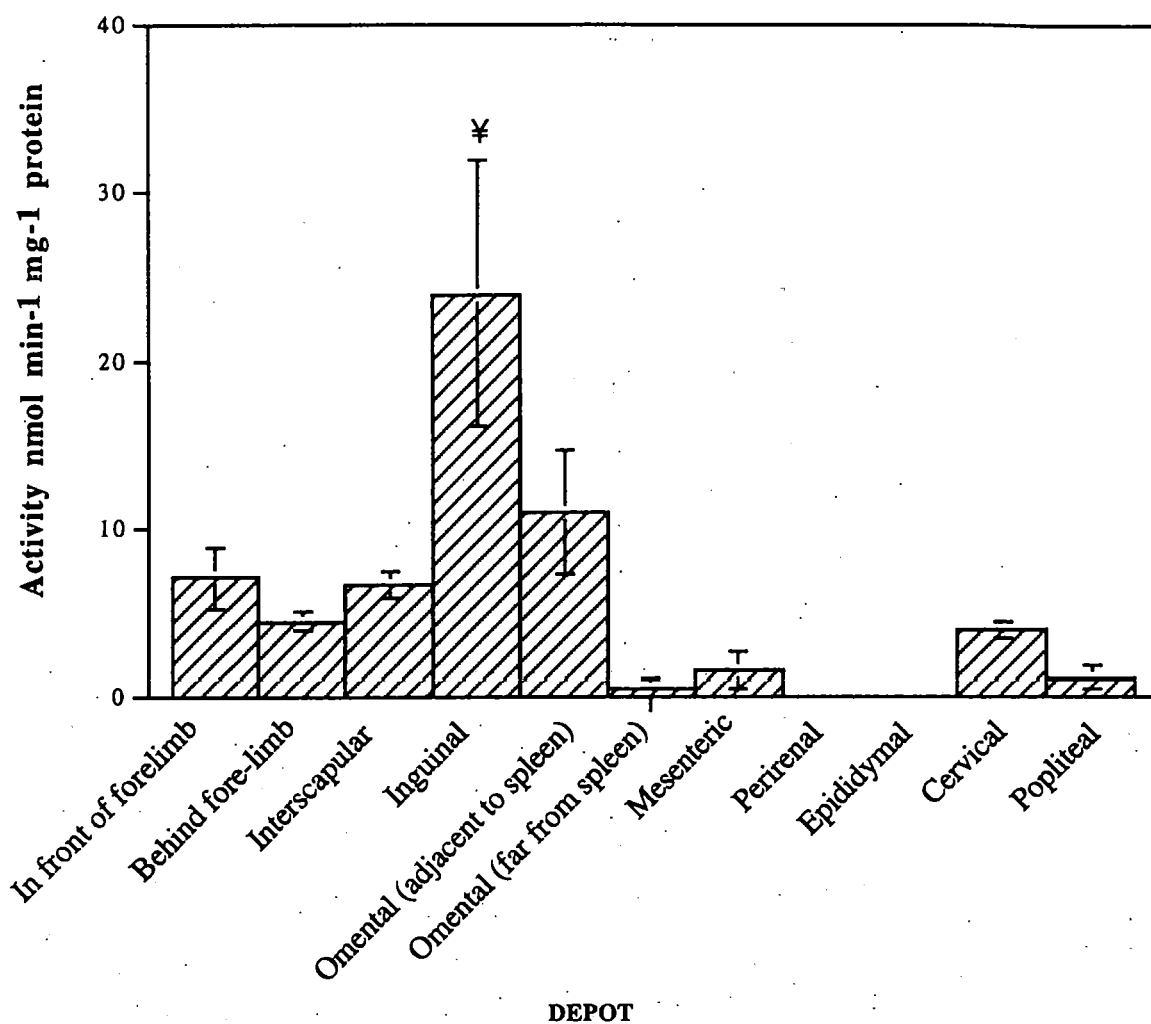


Figure 2.9 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein directly after exercise. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites.

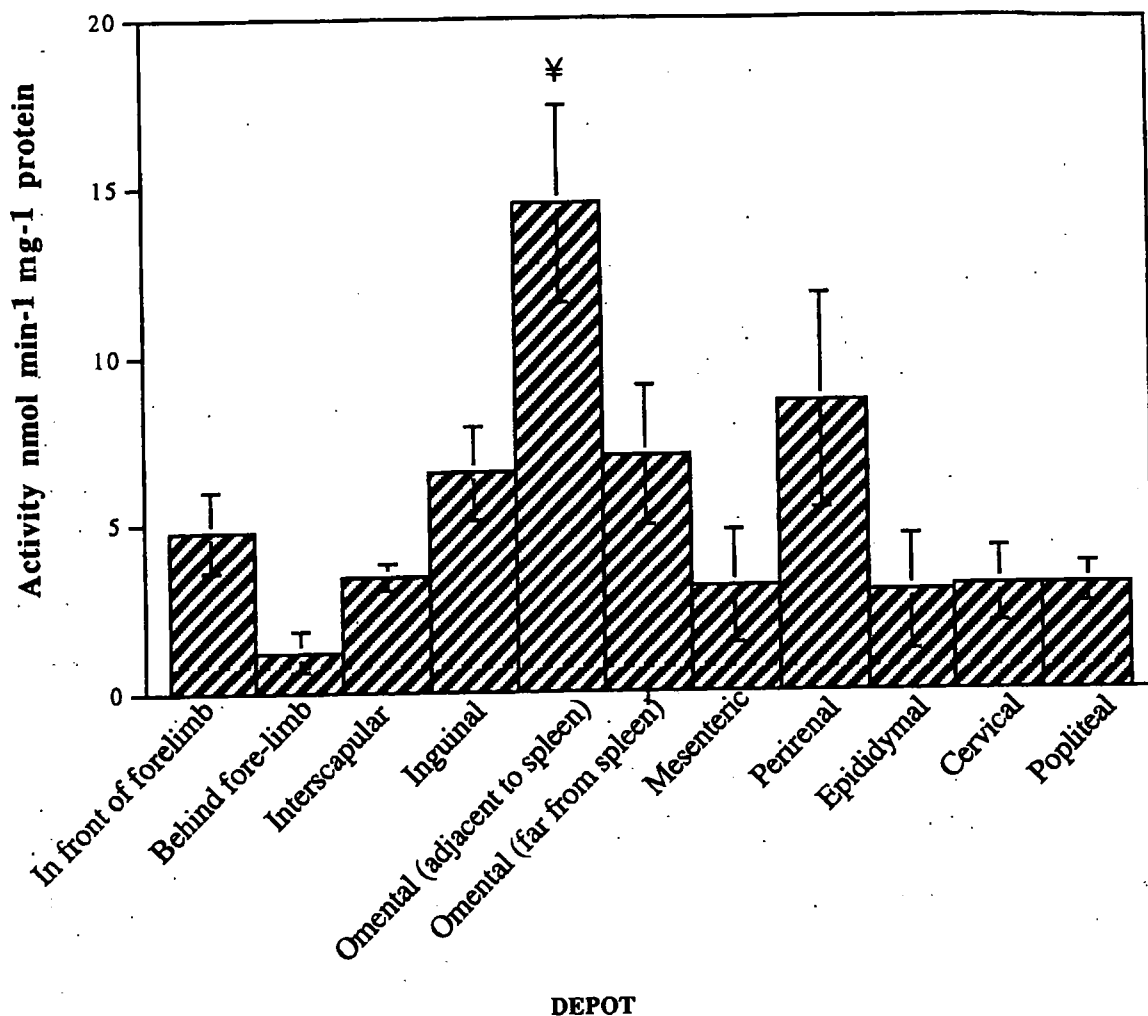


Figure 2.10 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein 30 minutes after exercise. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites.

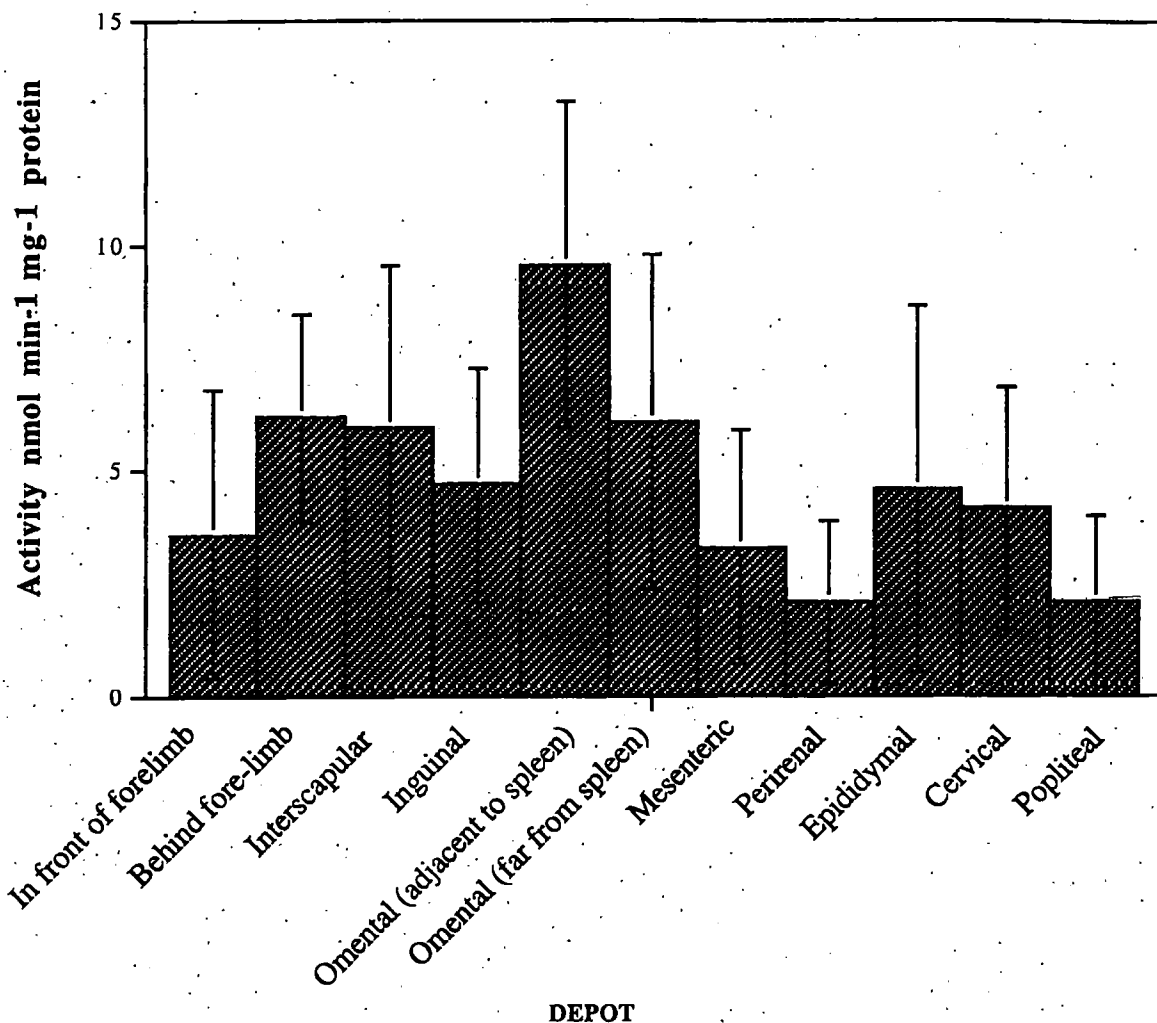


Figure 2.11 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein 60 minutes after exercise. There were no significant differences in values of enzyme activity between adipose sites.

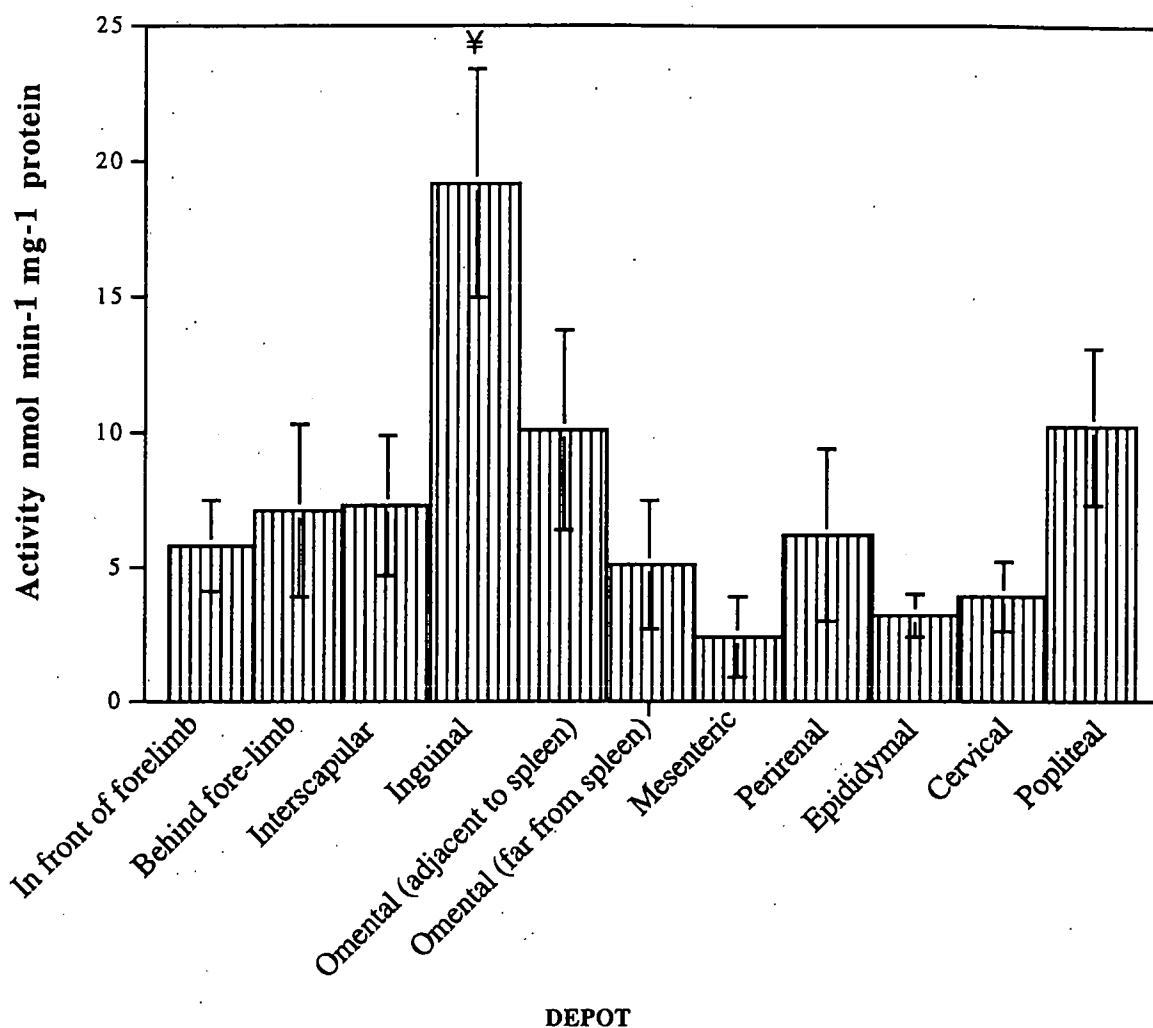


Figure 2.12 Glutaminase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹.mg⁻¹ protein 240 min after exercise. ¥ denotes glutaminase activity significantly higher ($P < 0.05$ or less) than all other sites.

2.4.10 The effects exercise over a time course of 240 minutes on glutaminase activity in different adipose sites

The results shown in Tables 2.5a, b and c show values of glutaminase activity after exercise over a time course, values of the fasted (sedentary) group are shown for comparison.

There was no measurable change in glutaminase activity at any time point after exercise in extracts of adipose tissue from the in front of fore-limb, interscapular, omental (adjacent to spleen) or mesenteric sites (Table 2.5a and b) compared with that of the sedentary, fasted values. Glutaminase activity was significantly reduced compared with the sedentary, fasted values directly following exercise in adipose tissue from the omental (far from spleen), and popliteal sites (Table 2.5b and c). In extracts of adipose tissue from the epididymal and perirenal site, glutaminase activity could not be detected directly following exercise (Table 2.5b). Enzyme activity remained reduced in epididymal and popliteal sites for up to 60 and 240 minutes respectively (Table 2.5b and c). Measurements of enzyme activity from the perirenal site appeared to fluctuate over the 240 minutes following exercise but values were only significantly different from those of the homologous depots of sedentary, fasted animals at 60 minutes after exercise. In adipose tissue from the inguinal and behind fore-arm sites, enzyme activity was significantly lower at 30 minutes following exercise than in sedentary, fasted guinea pigs. After 60 minutes, values of glutaminase activity remained lower in the inguinal site compared with the sedentary fasted values (Table 2.4a). In the cervical site, there was a small increase in enzyme activity directly after exercise which remained elevated for up to 240 minutes (Table 2.4c).

Table 2.5a Effect of exercise a time course of 240 minutes on glutaminase activity in extracts of superficial adipose tissue.

Values are mean (\pm SEM) expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet weight, and in parentheses nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein.

Fasted $n = 6$, exercised $n = 4$, n.d. = enzyme activity not detectable The statistical differences between the fasted and exercised means are denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Significantly higher ($P < 0.05$ or less) from most or all other sites measured in the same condition studied is denoted by ¥. Significantly lower ($P < 0.05$ or less) from most or all other sites measured in the same condition studied is denoted by •.

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
In front fore- limb	41.0 \pm 8.2 (6.4 \pm 1.1)	50.8 \pm 12.9 (7.1 \pm 1.8)	35.1 \pm 8.9 (4.8 \pm 1.2)	25.9 \pm 25.9 (3.6 \pm 3.2)	41.9 \pm 12.1 (5.8 \pm 1.7)
Behind fore- limb	56.1 \pm 13.4 (8.2 \pm 2.3)	34.2 \pm 4.7 (4.5 \pm 0.6)	8.8 \pm 4.8** (1.2 \pm 0.6**)	45.4 \pm 25.9 (6.2 \pm 2.3)	53.7 \pm 4.5 (7.1 \pm 3.2)
Interscapular	47.2 \pm 13.4 (7.5 \pm 2.8)	44.7 \pm 5.6 (6.7 \pm 0.8)	25.5 \pm 2.5 (3.8 \pm 0.4)	45.6 \pm 28.1 (6.0 \pm 3.6)	48.7 \pm 10.5 (7.3 \pm 2.6)
Inguinal	142.8 \pm 19.3 (33.0 \pm 4.0)	112.1 \pm 19.3 (24.0 \pm 7.9¥)	30.4 \pm 8.4*** (6.5 \pm 1.4***)	29.4 \pm 18.8*** (4.7 \pm 2.6***)	89.8 \pm 8.4 (19.2 \pm 4.2**¥)

Table 2.5b Effect of exercise over a time course of 240 minutes on glutaminase activity in extracts of intra-abdominal adipose tissue

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
Omental (adj. to spleen)	439.2±132.3 (14.9±4.2)	495.0±18.9 (11.0±3.7)	486.2±100.0 (14.5±2.9 [‡])	343.3±145.8 (9.6±3.6)	339.2±125.0 (10.1±3.7)
Omental (far from spleen)	30.5±4.3 (6.6±1.2)	2.4±2.4*** (0.5±0.5***)	55.3±16.9 (7.0±2.1)	49.6±34.2 (6.1±3.7)	40.3±4.3 (5.1±2.4)
Mesenteric	47.3±8.4 (3.0±1.0)	11.3±8.3 (1.6±1.1**)	22.3±12.5 (3.1±1.7)	20.3±17.7 (3.3±2.6)	17.2±10.5 (2.4±1.5)
Perirenal	72.1±12.8 (11.6±2.4)	n.d.	55.3±20.5 (8.6±3.2)	12.4±5.4** (2.1±1.8**)	40.0±20.1 (6.2±3.2)
Epididymal	92.2±19.3 (14.9±3.1)	n.d.	18.0±10.7** (2.9±1.7**)	27.4±27.4* (4.6±4.1**)	19.6±5.0* (3.2±0.8*)

Table 2.5c Effect of exercise over a time course of 240 minutes on glutaminase activity in extracts of intermuscular adipose tissue

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
Cervical	n.d.	34.5±4.7 (4.0±0.5)	26.3±7.5 (3.1±1.1)	35.0±26.3 (4.2±2.7)	33.9±11.4 (3.9±1.3)
Popliteal	79.5±18.9 (9.4±3.2)	7.6±4.4*** (1.1±0.7***)	20.7±4.2** (3.1±0.6**)	14.5±6.5** (2.1±1.9**)	69.1±19.4 (10.2±2.9)

2.5. Discussion

2.5.1 Preliminary study of the effect of fasting and feeding either a standard or 15% fat enriched diet on glutaminase activity in different adipose depots

2.5.2. Body mass of animals fed a standard or 15% fat enriched diet.

Guinea-pigs fed on the 15% fat experimental diet for 8 weeks did not differ in body mass compared with the animals fed on a standard diet. Both groups remained within 5% of their original mass at the beginning of the experimental period. The maintenance of a stable body mass with a high fat diet has previously been observed in adult guinea pigs and under some conditions in rats (Marchington and Pond 1990, Faust 1981). The introduction of the experimental diet over the 8-week training period suggests that either overall mass of food eaten was reduced and/or energy expenditure was increased to maintain a constant body mass.

2.5.3. Glutaminase activity in extracts of adipose tissue from the greater omentum, adjacent to the spleen of 12 month old guinea-pigs, after fasting and feeding either a 15% fat enriched or standard diet

Extracts of adipose tissue from the omental site, adjacent to spleen, had the greatest glutaminase activity when expressed g^{-1} wet weight under all conditions of this study. Furthermore, compared with the mean value of enzyme activity measured in the kidney, enzyme values in tissue extracts from the omental (adjacent to spleen) site were similar when expressed per g wet tissue weight and higher when expressed per mg of protein. This region of the omentum is thought serve as the first line of defence against peritoneal infection (Shimotsuma *et al.* 1993). It is a heterogenous mass of cells that include adipocytes surrounding numerous small clusters of lymphocytes and macrophages, termed milky spots (due to their white appearance under histological examination) and are analogous to regional lymph nodes but without the complex internal structure (Figure

2.13). Therefore, the omental (adjacent to spleen) tissue homogenates were not a pure preparation of adipocytes and included lymphatic tissue. However, this explanation is unlikely for the omental (far from spleen) as histological examination shows adipose tissue taken from this area of the omentum as a homogenous mass of small adipocytes (Figures 2.14) nor for inguinal, which contains a mass of large adipocytes (Figure 2.15). (Histochemistry for these photomicrographs performed by C. Elcoate, 1995).

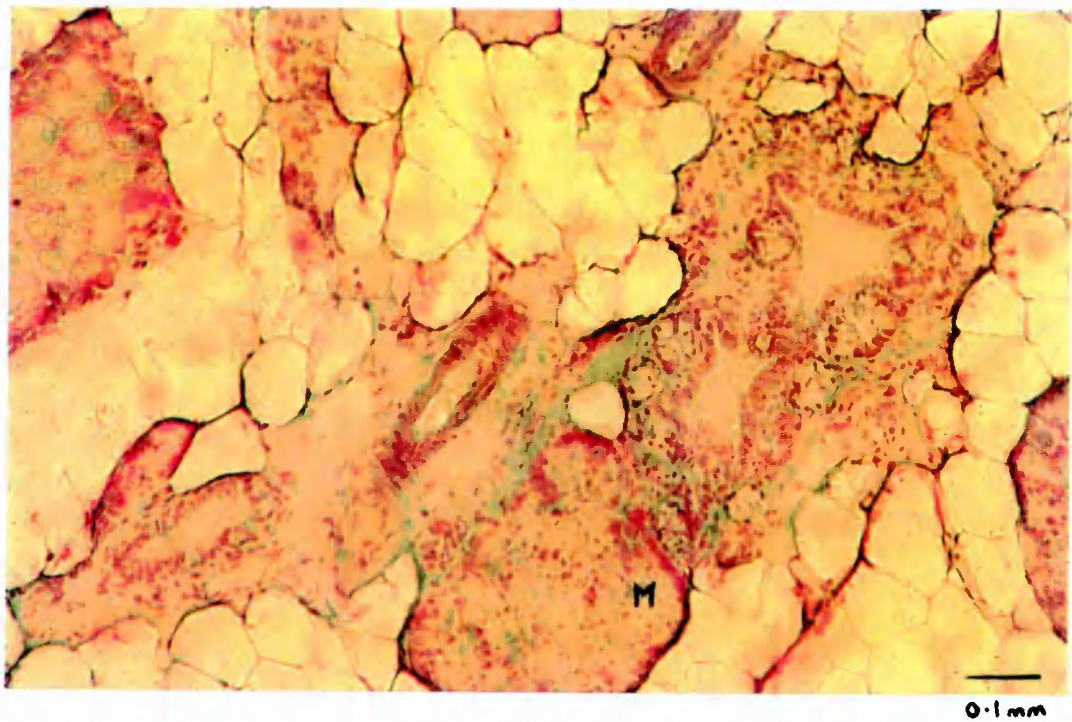


Figure 2.13 Section of adipose tissue taken from the omentum adjacent to spleen site (100 x) showing adipocytes surrounding dense masses of macrophages termed 'milky spots' (M). Haemotoxylin-eosin-azure stain.

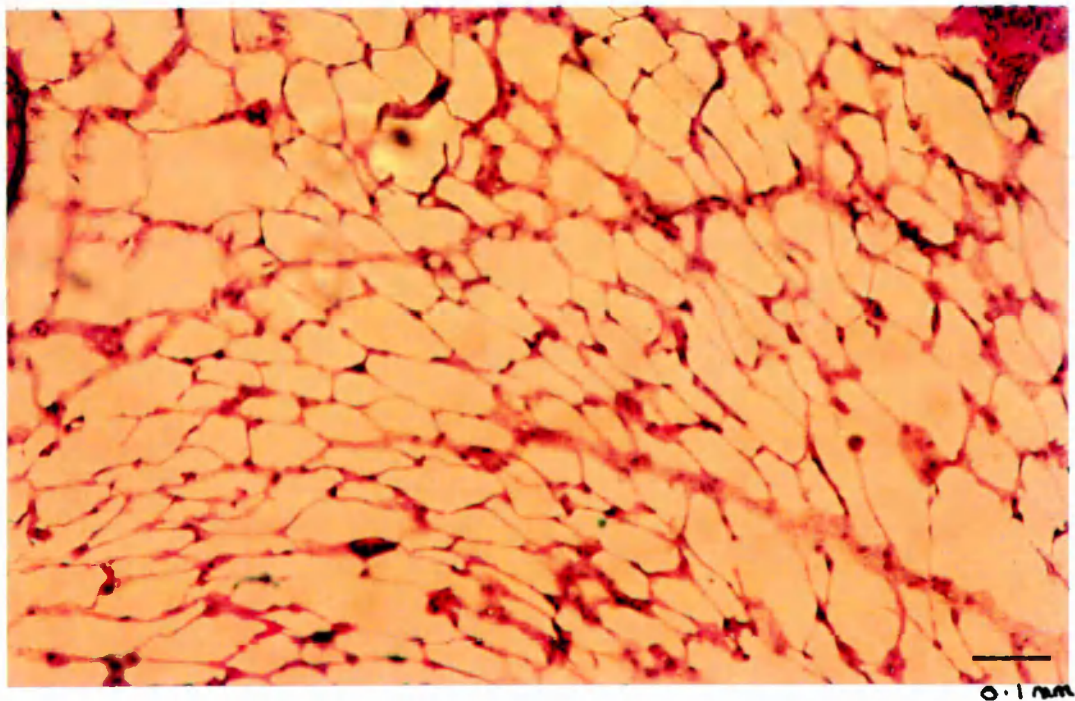


Figure 2.14 Section of adipose tissue taken from the omentum (far from spleen) site (100x) showing numerous small adipocytes.

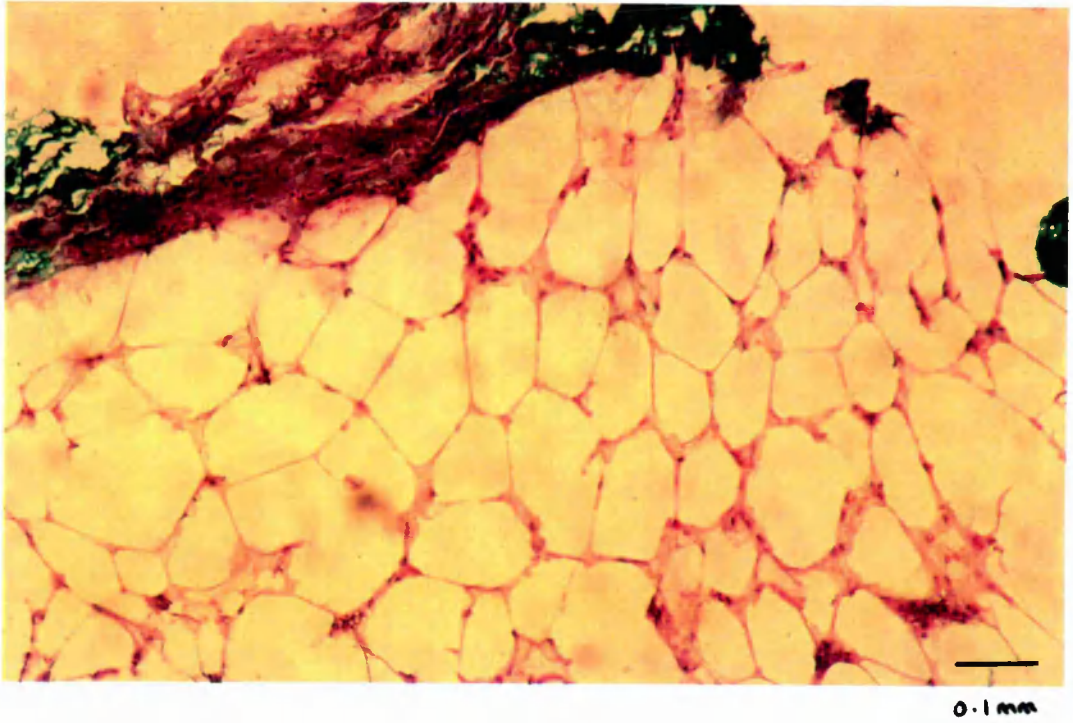


Figure 2.15 Section of adipose tissue taken from the inguinal site (100x) showing numerous large adipocytes.

Lymphatic tissue consumes high quantities of glutamine through the action of glutaminase (Ardawi and Newsholme 1984b). Therefore, the high capacity for this region of the omentum to utilise significant quantities of glutamine is probably due to the lymphatic tissue present amongst the adipocytes (Figure 2.12). The anatomical position of the omentum enables it to utilise large quantities of glutamine as the ammonia produced from its deamidation can readily diffuse into the portal blood system to be exported directly to the liver, via the superior mesenteric and splenic vein.

The omentum in adult male guinea-pigs represents approximately 4% of total dissectable adipose tissue (Figure 2.5). Therefore, this section of a relatively small depot (in mass) has the capacity to hydrolyse significant quantities of glutamine. The maximal glutaminase activity measured in this site was $3 \mu\text{mol min}^{-1} \text{g}^{-1}$ wet tissue (Table 3.2), which is comparable with that of other tissues known to utilise glutamine at a high rate in the guinea-pig, such as kidney, $4.8 \mu\text{mol min}^{-1} \text{g}^{-1}$ wet tissue (Table 2.2), and mesenteric lymph nodes, maximal activity $6 \mu\text{mol min}^{-1} \text{g}^{-1}$ wet tissue (Ardawi and Newsholme 1984b). These values of enzyme activity measured in this site are nonetheless high considering that approximately 50% of the tissue in this area of the omentum consists of adipocyte triacylglycerols. As glutamine metabolism in this site has not been previously measured, no account of it has been taken when calculating glutamine utilisation by the gut and splanchnic bed (Windmueller and Spaeth 1974). Furthermore, previous estimates of glutamine extraction by the spleen have been measured by catheterising the splenic vein, which also drains the omentum, therefore may have attributed a falsely high value of glutamine hydrolysis by this organ (Deutz *et al.* 1992). The finding from this study that this region of the omentum, albeit a relatively small site in mass, has a significant capacity to deaminate glutamine underlines the importance of including measurements from different adipose sites to obtain a realistic view of its contribution to whole body glutamine metabolism.

In order to apportion values of glutaminase activity between the adipocytes and lymphatic tissue in this region of the omentum, it will be necessary to isolate these cell types and determine glutaminase activity *in vitro*. Nonetheless, the data from this study of

glutaminase activity in the omentum represents a novel finding in that this site has not been previously considered when investigating whole body glutamine metabolism, and adds to the list of tissues which hydrolyse significant quantities of this amino acid.

Glutaminase activity was significantly lower in extracts of omental tissue adjacent to spleen from animals fed the 15% fat-enriched diet compared with tissue from the group fed the standard diet. As the lymphatic tissue contained in this section of the omentum was not separated from adipose tissue, the reduction in enzyme activity cannot be apportioned to either tissue type. However, glutaminase activity in pure adipose tissue taken from the omentum (far from spleen) was higher in the group fed a 15% fat-enriched diet, than in samples from the standard diet fed group. Therefore, it is probable that reduced activity in tissue from adjacent to spleen was attributable to a reduction in glutaminase activity in the lymphatic tissue located in this site. This suggestion is in agreement with a study by (Costa Rosa *et al.* 1993) which demonstrated alterations in glutaminase activity in macrophages from rats fed a 15% fat diet for 6 weeks.

2.5.4. Glutaminase activity in extracts of adipose tissue from sites other than omental adjacent to spleen, in 12 month old guinea-pigs, after fasting and feeding a 15% fat enriched or standard diet

Glutaminase activity measured in extracts of pure adipose tissue from inguinal site in the fasted animals had the second highest value of glutaminase activity when expressed per g wet tissue weight and was higher than those of all other sites when expressed per mg protein ($P < 0.05$). The high glutaminase activity in this adipose tissue from this site indicates that in contrast to its relatively low capacity to metabolise lipids (refer to Table 1.1, Chapter 1) this relatively large depot has the capacity to hydrolyse significant quantities of glutamine during fasting. Glutaminase activity, per g wet weight, in the other superficial sites and in the omental (far from spleen) site from the fasted animals also had relatively high values of glutaminase activity compared with the remaining intra-abdominal and intermuscular sites in which values of enzyme activity were relatively low or undetectable. During fasting, the superficial and omental sites could therefore, through the

action of glutaminase, play an active role in the extraction of glutamine from the circulation and its subsequent hydrolysis.

After 30 minutes of feeding, glutaminase activity was significantly increased in superficial adipose tissue from the inguinal and interscapular sites. Measurements taken from the in front of fore-limb and behind fore-limb sites also indicated an increase in enzyme activity but the relatively high standard errors precluded statistical significance. In extracts of adipose tissue from all the intra-abdominal sites and the intermuscular sites there was also an increase in glutaminase activity after feeding, regardless of diet type. In this study, post-prandial measurements of glutaminase activity were taken at the end of the 30 minute feeding period which correlates with the time at which absorbed amino acids, would be expected to be appearing in the circulation (Elia *et al.* 1989). In the rat, it is thought that a substantial amount of dietary glutamine is utilised by cells of the intestine with little entering the bloodstream (Windmueller 1982). However, studies on humans using radioactively labelled glutamine have shown that when administered orally, 40 to 50% enters the peripheral circulation (Matthews 1990, Déchelotte *et al.* 1991). To the author's knowledge, glutamine absorption has not been studied in the guinea pig after feeding, therefore it cannot be ruled out that in this animal, dietary glutamine may enter the circulation along with other amino acids after a meal.

It is not known whether adipose tissue contributes significantly to the disposal of circulating glutamine after a meal. Previous *in vivo* studies in humans and in rats have shown that adipose tissue releases glutamine in the fed state (Frayn *et al.* 1991 and Kowalski and Watford 1994a). However, the results from these studies were obtained by measuring arteriovenous differences, which reflect a net exchange across the tissue, therefore simultaneous uptake of glutamine may occur without being detected. The physiological role of glutamine metabolism in adipose tissue depots after a meal is yet to be elucidated. However, the anatomical location of the omentum and associations with tissues which also utilise glutamine i.e. the gut and lymphoid tissue could allow a cycling of glutamine within adipose tissue and release to local tissues/organs. Whereas large

superficial depots such inguinal could be of benefit to whole body glutamine balance as it may be released into the general circulation.

Glutaminase activity measured in the inguinal and cervical sites taken from the animals fed a 15% fat enriched diet was significantly reduced compared with that measured in these sites from animals fed the standard diet. The post-prandial increase in glutaminase activity observed under standard diet conditions, in the above mentioned sites, therefore suggests that adipose tissue as a whole increases its capacity to hydrolyse glutamine but that this capacity is reduced in certain sites with a diet that is lipid enriched.

2.5.5 Site-specific effect of fasting, feeding and exercise on glutaminase activity in adipose tissue over a time course of 240 minutes

2.5.6 Glutaminase activity in adipose tissue from fasted animals

As with the previous study, extracts of adipose tissue from the omental site (adjacent to spleen) had the greatest activity of glutaminase g^{-1} wet tissue weight, as discussed in Section 2.5.2. It is likely that glutaminase activity measured in tissue extracts from this site was mostly attributable to lymphatic tissue as enzyme activity was considerably less in extracts of pure adipose tissue taken from the 'far from spleen' site. Also in agreement with the previous study, extracts of adipose tissue from the inguinal depot had the greatest activity (apart from omental, adjacent to spleen), in the fasted condition.

2.5.7. Changes in glutaminase activity in different adipose depots after feeding over a time course of 240 minutes

The data demonstrate that after feeding, the capacity of adipose tissue to hydrolyse glutamine over the time course was enhanced through increased glutaminase activity in all depots studied. Furthermore, there were rapid, site-specific changes in enzyme activity over the time course studied. The response to feeding was broadly similar in all adipose sites measured in that there was an increase in enzyme activity at 30 minutes after the

feeding period, a time at which insulin along with glucose and dietary absorbed amino acids would be expected to be present at higher concentrations in the circulation (Elia *et al.* 1989). At 60 minutes after the feeding period, there was no measureable enzyme activity in any of the sites studied, which coincides with the expected appearance of absorbed dietary lipids in the bloodstream (Coppack *et al.* 1990).

Modulation of glutaminase activity at a subcellular level *in vivo* could be controlled by either alterations in the rate of its synthesis or degradation, or by changes in the existing complement of glutaminase. The properties of adipose tissue glutaminase have not been well characterised. However, as kidney-type glutaminase mRNA has been identified in rat adipose tissue (Watford 1991) it is assumed that the enzyme structure is similar. Monomeric, dimeric and tetrameric forms of glutaminase have been identified from renal mitochondrial preparations, with the tetrameric form being active in the presence of saturating concentrations of phosphate (Curthoys *et al.* 1984). Renal glutaminase, in the active form, is a heterotetramer composed of three 66-kDa subunits and one 68-kDa subunit, the latter possibly acting to retain the enzyme within the mitochondrial matrix (Haser *et al.* 1985)]. Other more physiologically relevant factors which may regulate adipose tissue glutaminase *in vivo* may include fatty acids as previous workers have shown that fatty acyl-CoA derivatives in low concentrations activate glutaminase *in vitro* whereas, long chain fatty acyl-CoA derivatives at high concentrations are effective inhibitors of purified pig kidney and brain glutaminase (Kvamme and Torgner 1974; 1975). A similar finding using rat kidney cortex tubules was also reported by Baverel *et al.* 1984. The effects of different chain length saturated and unsaturated fatty acids have not to the author's knowledge been investigated in relation to adipose tissue glutaminase activity *in vitro* and represents a potentially interesting avenue of research, especially as it indicates a link between lipid metabolism and amino acid metabolism in the adipocyte.

Characterisation of the mitochondrial processing of glutaminase in the kidney has established that increases in activity result from increased protein synthesis rather than from processing of the enzyme (Srinivasan *et al.* 1995). Abundance of glutaminase

mRNA has been shown to increase in the kidney during metabolic acidosis, but this process is relatively slow, occurring over a period of days rather than hours (Watford 1991). However, under the same conditions, although high levels of glutaminase mRNA were detected in adipose tissue, no changes in abundance of the enzyme were observed (Watford 1991). The rapid alterations in the activity of glutaminase after feeding demonstrated in this study suggest that enzyme regulation in adipose tissue after feeding is controlled by different processes from those in the kidney. Changes in activity could therefore be due to either post-translational modification and/or the efficiency of translocation.

Hepatic glutaminase activity has been shown to be rapidly altered by hormones such as glucagon, and catecholamines, but the mechanism by which these effects occur remains unclear. It has been suggested that changes in glutaminase activity may be a result of alterations in intracellular calcium concentrations and/or phosphorylation of cytoplasmic proteins activated by protein kinases (Brosnan *et al.* 1995).

It is also possible that regulation of a pre-existing form of glutaminase in adipose tissue could be mediated by the anabolic effects of hormones such as insulin which is in itself stimulated by protein ingestion and is present in the circulation during and after feeding. Uptake of the amino acid analogue, α -aminoisobutyric acid in isolated mouse epididymal adipocytes has been shown to be stimulated by insulin (Touabi and Jeanrenaud 1969). However, this study suggested that alterations in amino acid metabolism in adipose tissue were due to intracellular changes in the concentrations of free fatty acids and ATP induced by the action of insulin, rather than a direct effect of insulin on the transport mechanism.

Plasma insulin in humans and rats is significantly elevated within 30 minutes after the onset of feeding, with concentrations remaining elevated for up to 90 minutes (Coppack *et al.* 1992, Strubbe *et al.* 1977, West *et al.* 1989). The changes in glutaminase activity observed in this study at directly after, and 30 minutes after the feeding period, correlate well with the time course of insulin release into the circulation and the short term intracellular action of this hormone (Denton *et al.* 1981).

There were site-specific differences in the response to feeding over the time course. The greatest increases in maximal glutaminase activity directly after the feeding period were observed in the portally drained sites, omental (far from spleen), and mesenteric, as well as the intermuscular cervical depot. The omental site (adjacent to spleen) also demonstrated an increase, albeit smaller, in enzyme activity directly after feeding. Measurements of glutaminase in adipose tissue from the remaining sites directly after the feeding period showed either no change or a decrease in activity. There was an increase in enzyme activity 30 minutes after feeding in adipose tissue from the in front of fore-limb and behind fore-limb, interscapular, inguinal and popliteal sites indicating that these depots may become involved in the metabolism of circulating glutamine.

No measurable glutaminase activity could be detected 60 minutes after the feeding period in extracts of adipose tissue from any of the sites, with the exception of omental (adjacent to spleen) which, as discussed previously, may be due mainly to the lymphatic tissue in this region rather than the adipocytes. It is noteworthy that adipose tissue extracts from this site demonstrated the greatest enzyme activity at this time point after feeding when glutaminase activity could not be detected in any of the other sites containing pure adipose tissue. It is therefore probable that the enzyme activity measured was due to the lymphatic rather than the adipose tissue. The apparent deactivation of glutaminase in certain adipose tissue extracts 60 minutes after feeding suggests a fast and highly regulated control of the enzyme in adipose tissue. The inability to detect glutaminase activity *in vitro* at 60 minutes after feeding could be due to either rapid degradation or covalent modification of the enzyme *in vivo*. Factors which may modify glutaminase activity in adipose tissue have not previously been studied but could include fatty acids, which are known to reduce glutamine flux through down regulation of glutaminase activity in the kidney *in vitro* (Baverel *et al.* 1984). Furthermore, as mentioned previously, it has also been shown that different chain length fatty acyl CoA derivatives inhibit glutaminase *in vitro*, particularly when the fatty acyl chain is elongated (Kvamme and Torgner 1974; 1975).

The data shown in Table 2.2 are consistent with this idea, as the study of animals fed a fat enriched diet, which is likely to be associated with high levels of circulating lipids, had the effect of lowering glutaminase activity in adipose tissue compared to that of the animals fed a standard diet. The mechanism by which this inhibition occurs is not known. Baverel *et al.* (1984) proposed that glutaminase activity could be inhibited by the accumulation of glutamate due to a shift in the mitochondrial NAD/NADH ratio towards reduction, secondary to fatty acid oxidation. This situation would result in reduced flux through glutamate dehydrogenase and an increase in the concentration of glutamate.

There have been no comparable time course studies on glutaminase activity after feeding in different adipose tissue sites, and the site-specific differences reported in this study could be due to a number of factors. Different values of enzyme activity could be attributable either to cell volume and numbers, or to protein content of the different adipose sites. However, site-specific differences in activity were significant and consistent regardless of whether activity was expressed g^{-1} wet weight or mg^{-1} protein. This comparison indicates that site-specific differences were not attributable either to differences in cell volume, or to the protein content of the adipose sites. Other reported site-specific differences in enzyme activity in adipose tissue, namely lipoprotein lipase, hexokinase and phosphofructokinase also cannot be consistently related to cell volume *per se* (Pond and Mattacks 1987; Pond *et al.* 1992).

The delivery of circulating glutamine to adipose tissue may have an indirect site-specific effect on glutaminase activity by altering the availability of this substrate to the enzyme. The mesenteric adipose tissue of the fasted rat has been shown to have the highest blood flow *in vivo* compared with other major adipose depots (West *et al.* 1989). In the present study, mesenteric adipose tissue, which shares a blood supply with the gut, would be first in line to respond to, and sequester, absorbed amino acids before they reach the liver. However, in the study by West *et al.* (1989) blood flow was uniformly decreased in all depots after feeding for up to 45 minutes. As glutaminase activity was differentially altered in adipose tissue after feeding, blood flow is unlikely to be a primary factor contributing site-specific changes in enzyme activity observed in this study.

As discussed previously, it is likely that the alterations in glutaminase activity are hormonally mediated in adipose tissue. Site-specific differences in the responsiveness of rat and human adipose tissue to hormones such as insulin, the catecholamines and growth hormone have been reported (reviewed by Leibel *et al.* 1989). The antilipolytic effect of insulin has been shown to be greater in subcutaneous than in omental adipocytes of humans, and omental and mesenteric adipocytes of guinea pigs (Bolinder *et al.* 1983 and Pond and Mattacks 1991). It is suggested that regional variations in the action of insulin occur at the post-receptor binding level but its mechanism has yet to be elucidated. The physiological basis for such differences in adipose tissue metabolism may be of importance to lipid and amino acid acquisition and/or mobilisation, particularly in sites which have direct access to the portal system. The site-specific alterations in glutaminase activity over the time period after a meal may also in part be due to differences in the responsiveness of adipose depots to the action of insulin (discussed in Chapter 3).

2.5.8. Changes in glutaminase activity in different adipose depots after exercise

As with feeding, there were site-specific differences in glutaminase activity after exercise over the time course of 240 minutes. In adipose tissue from omental (far from spleen), epididymal, perirenal and popliteal sites, glutaminase activity was virtually abolished directly after exercise compared with the values for the sedentary fasted and fed animals. Conversely, there was a slight, but significant, increase in activity in adipose tissue from the cervical site, which remained elevated for up to 240 minutes after exercise. Values of enzyme activity returned to those measured in the sedentary fasted state after 30 minutes in adipose tissue from the omental (far from spleen). However, glutaminase activity in tissue taken from the epididymal, perirenal and popliteal sites remained low until up to 60 minutes after exercise. The behind fore-limb and inguinal sites were slower to respond to exercise, with a decrease in activity after 30 minutes which was maintained after 60 minutes in inguinal tissue only. This relatively weak response by the inguinal site is in line with the small alterations in lipid metabolism after exercise (refer to Table 1.1, Chapter 1).

but is in contrast to the much greater alterations in glutaminase activity in response to feeding by this site. In all of the above mentioned sites, glutaminase activity returned to fasted values by 240 minutes. The remaining sites measured, in front of fore-limb, interscapular and omental (adjacent to spleen), again in sharp contrast to the response found after feeding, showed little or no change in enzyme activity over the time course studied.

Under the conditions of brief, acute exercise used in this study, site-specific changes in glutaminase activity may be affected by a differential response by depots to circulating hormones associated with exercise or stress. The guinea-pigs used in this study were normally sedentary and had not previously been exposed to the exercise regime employed in this study. Therefore, such a short, acute form of exercise, would probably increase levels of stress hormones such as ACTH and noradrenaline. Site-specific differences in catecholamine-mediated lipolysis have been reported (Leibel *et al.* 1989). In human omental adipose tissue, noradrenaline-stimulated lipolysis is more pronounced than in subcutaneous adipocytes of a similar size (Östman *et al.* 1979). More recently, it has been shown that the pattern of protein expression differs according to anatomical site, with a greater concentration of β_3 -adrenergic receptor mRNA in intra-abdominal compared with inguinal adipose tissue (Cousin *et al.* 1993). Amino acid metabolism in adipose tissue has also been shown to be affected by adrenaline and ACTH. These hormones inhibit uptake of the amino acid analogue α -aminoisobutyric acid in isolated mouse epididymal adipocytes (Touabi and Jeanrenaud 1969). As in muscle, this effect may be mediated by β -adrenergic receptors and dibutyryl cyclic AMP. The effect of exercise on glutaminase activity could therefore serve to decrease the capacity for glutamine breakdown in adipose tissue depots under physiological conditions where this amino acid may be required as a source of nitrogen for the synthesis of amino acids in other tissues such as muscle. In addition, stimulation of lipolysis via the sympathetic nervous system in adipose tissue and the subsequent release of free fatty acids may also serve to inhibit its metabolism of amino acids.

2.5.9. Conclusions

From the data presented in this study the capacity for glutamine hydrolysis through the action of glutaminase, post-prandially and post-exercise, over the time course, indicate that the enzyme is subject to acute regulation and there is evidence to suggest that it may be integrated with that of lipid metabolism within the adipocyte.

Site-specific differences in the assimilation and mobilization of lipids have been demonstrated in humans and rodents (Mattacks *et al.* 1987, Mattacks and Pond 1988, Pond and Mattacks 1991, Östman *et al.* 1979, Vikman *et al.* 1996 and Arner 1995). It has been suggested that the intra-abdominal depots (omental and mesenteric) and intermuscular (popliteal and cervical) are more responsive to short-term lipid storage and release, whereas, the larger superficial depots (and epididymal in rodents) are of greater importance for long term lipid storage (Rebuffé-Scrive *et al.* 1987, West *et al.* 1989, Mattacks *et al.* 1987 and Pond *et al.* 1992). The data presented in this study demonstrate that, as with lipid and carbohydrate metabolism, there are site-specific differences in glutamine metabolism. During fasting, the omental (adjacent to spleen) and the inguinal site had the greatest capacity to utilise glutamine. After feeding, glutaminase activity was increased in all adipose sites studied. The portally drained omental (far from spleen) and mesenteric, and the intermuscular cervical sites showed the greatest increase in glutaminase activity directly after a meal. With exercise, glutaminase activity was significantly decreased in adipose tissue from the intra-abdominal omental (far from spleen), epididymal, perirenal sites and in the intermuscular popliteal site. These site-specific changes in glutaminase activity with altering physiological conditions may be due to intrinsic differences in adipose tissue glutamine metabolism. These site-specific differences may also have an effect on the flux of glutamine through different adipose depots, which could be of functional importance to the physiological basis for glutamine metabolism in adipose tissue.

Chapter 3

**The effects of insulin,
dexamethasone and isoprenaline on
glutamine uptake in isolated
adipocytes prepared from five
different adipose depots**

3.1 Introduction

Glutamine is a multifunctional amino acid which, as well as being a component of proteins, is a substrate for a variety of metabolic pathways in different tissues. Therefore, glutamine supply to these competing tissues must be regulated according to their specific metabolic requirement for this amino acid with changing physiological status. Maintenance of whole-body glutamine homeostasis under different physiological conditions involves, in part, alterations in the capacity of utilising tissues and organs to extract this amino acid from the circulation (Welbourne 1987). Effectors of glutamine uptake *in vitro* differ between tissues and reflect their distinct metabolic requirements with altering physiological conditions. Changes in transmembrane fluxes of glutamine which modify uptake or release by tissues play an important role in regulating whole-body glutamine flow and can serve to divert the supply of this amino acid to utilising tissues. Glutamine transport systems identified in membranes of muscle, liver and kidney appear to be tissue-specific which in turn could match their different metabolic requirements for glutamine (Christensen 1990).

Evidence for sodium-dependent and sodium-independent glutamine transport mechanisms has been derived from studies on rat hepatocytes (Kilberg, 1980) and sarcolemmal vesicles from human skeletal muscle (Ahmed *et al.* 1993). Uptake of glutamine in rat hepatocytes occurs exclusively by a Na^+ -dependent system which is insensitive to stimulation by either insulin or glucagon (Ahmed *et al.* 1993). Glutamine uptake into muscle is thought to be predominantly facilitated by a sodium plus glutamine transporter (referred to as N^{m}) which is insulin-sensitive (Tadros *et al.* 1993). (The designation 'N' refers to a hepatic transport system serving for glutamine, histidine and arginine. The term ' N^{m} ' has been applied for a muscle transport system largely specific for glutamine, but the superscript m implies as yet no generic relation to hepatic system N). Therefore, the influx of glutamine into muscle can be modulated by both the action of insulin and changes in the circulating concentrations of glutamine.

There is no information on the mechanism(s) of glutamine transport into adipose tissue but there is some evidence that it may share some similarities to that of muscle.

Uptake of the non-metabolisable amino acid analogue α -aminoisobutyric acid into mouse epididymal isolated adipocytes has been shown to be stimulated by insulin, and inhibited by adrenaline and ACTH (Touabi and Jeanrenaud 1969). Furthermore, Kowalchuk *et al.* (1988) demonstrated that the rate of glutamine utilisation in epididymal isolated rat adipocytes was increased when insulin (10 mU ml⁻¹) and glucose (1 mM) were added to the incubation medium.

The study of glutaminase described in the previous Chapter shows alterations in enzyme activity in adipose tissue extracts with fasting, feeding and exercise. In order to evaluate whether alterations in glutaminase activity with different physiological conditions correlate with alterations the intracellular availability of glutamine, the present study described in this Chapter measured the effects of insulin, isoprenaline and dexamethasone on the uptake of glutamine by living isolated adipocytes prepared from different adipose depots of the guinea-pig.

3.1.1. Hypothesis

The uptake of glutamine into adipocytes differs between depots and is (in part) regulated by the hormones released with feeding, fasting or exercise that may have site-specific effects on different adipose depots. Therefore, alterations in the uptake of glutamine and/or associated changes in the catabolic activity of glutaminase in different adipose sites may affect the depot's capacity to hydrolyse glutamine. These properties determine the part played by adipose tissue in maintaining whole body and local glutamine homeostasis with altering physiological status.

3.1.2. Aims

The aims of this study were to determine the effects of different hormones or their analogues on glutamine uptake into adipocytes isolated from five selected adipose sites which demonstrated major site-specific responses in glutaminase activity with feeding, fasting and exercise described in Chapter 2. The hormones/analogues used were; Bovine

insulin, dexamethasone (a glucocorticoid analogue) and isoprenaline (a β -adrenergic agonist).

3.1.3. Study design

Bovine insulin was used in this study as it has shown to be the most effective of a variety of commercially available insulins in guinea-pig adipocytes (Pond and Mattacks 1991). Isoprenaline, a noradrenaline analogue, was used in this study as it is chemically stable during a 60 minute incubation period. Furthermore, guinea-pig adipocytes exhibit a strong β -adrenergic-mediated lipolytic response to this β -adrenoceptor agonist (Lafontan and Berlan 1993). It was used in the present study as it was hoped that it may have some effect on adipocyte glutamine metabolism.

Dexamethasone, a glucocorticoid analogue, was used in this study as it is known to be more stable and longer acting *in vitro* than cortisol. Dexamethasone administration in rats has been shown to increase glutamine utilisation by the small intestine *in vivo* and glutaminase activity in intestinal mucosal cells *in vitro* (Souba 1993b and Ardawi and Newsholme 1988). The aim of this study was to determine whether dexamethasone alone had any effect on glutamine uptake into isolated adipocytes from different sites and whether dexamethasone potentiated the effect of insulin on glutamine uptake into isolated adipocytes. Serum cortisol was also measured in fasted and fed animals to ascertain the circulating levels of this hormone in these two conditions.

3.2. Materials and methods

3.2.1. Animals and Dissection Procedure

Adipose tissue was obtained from 6 month old male Bolivian guinea-pigs (body mass 865 ± 32 g), born and raised at the Open University. The animals were fasted for 18 hours overnight then sacrificed between 0830 and 0930 h with an intra-peritoneal injection of 3% pentobarbital dissolved in phosphate buffered saline. Approximately 1 g samples of adipose tissue were then dissected immediately from the following five selected sites

which were chosen according to site-specific differences in glutaminase activity measured in adipose tissue extracts after feeding fasting and exercise described in chapter 2.

Subcutaneous - Inguinal. Chosen due to the relatively high glutaminase activity with fasting and in the early post-prandial period (see Table 2.3, Chapter 2).

Intra-abdominal - Omental (far from spleen), mesenteric, and epididymal. The omental site was chosen due to the large changes in enzyme activity observed after feeding and exercise (see Table 2.3, Chapter 2). The mesenteric site was chosen because of its relatively low activity with fasting and its alterations in activity after exercise (see Table 2.3, Chapter 2). The epididymal site was chosen as the response to feeding in this site was similar to that of the perirenal site but had a greater response to exercise than the latter (see Table 2.3, Chapter 2).

Intermuscular - Popliteal. This site was chosen to represent intermuscular adipose tissue as it responded to feeding and exercise in a similar way to that of the cervical site (see Table 2.3, Chapter 2).

The dissection procedure took approximately 5 to 10 minutes.

Epididymal adipose tissue was used for the preliminary experiments to establish the time course of glutamine uptake in isolated adipocytes.

3.2.2. Chemicals

All chemicals and enzymes are detailed in Appendix II

3.2.3. Preparation of buffers

Buffers were prepared on the day of the experiment and consisted of the following:

Krebs-Ringer Bicarbonate buffer (KRB) (pH 7.4):

Chemical	Concentration (mM)
NaCl	136
KCl	2.54
Mg SO ₄ . 7 H ₂ O	1.18
KH ₂ PO ₄	1.18
NaHCO ₃	16.0
CaCl ₂	1.3

The buffer was gassed (O₂, 95%/CO₂, 5%) and incubated at 37°C for 30 minutes in a waterbath. The buffer was then divided into wash buffer, preincubation and incubation buffer. The wash buffer consisted of KRB supplemented with BSA (0.2%) and glucose (5.6 mM), pH 7.4. The preincubation and incubation media consisted of KRB and BSA (0.2%) with glucose (1 mM), pH 7.4. The incubation media were then supplemented with glutamine (2 mM), and L-[¹⁴C(U)] glutamine (0.3 µCi ml⁻¹).

3.2.4. Preparation of hormones and analogues.

Preparation of hormones are detailed in Appendix II

3.2.5. Preparation of adipocytes

Isolated adipocytes were prepared by a method modified from that of Rodbell (1964). One gram samples of adipose tissue from each of the above mentioned sites were weighed and

placed immediately into siliconised 30 ml glass vials containing 10 ml of Hanks balanced salt solution (HBSS) with 1.5% BSA pre-warmed to 37°C. The tissue samples were chopped with scissors into approximately 1 mm³ pieces. The vials containing the adipose tissue samples were placed in a water bath at a temperature of 37°C. The appropriate concentration of collagenase, detailed below, was then added to the samples.

Preliminary experiments indicated that samples of adipose tissue from different sites required different concentrations of collagenase to obtain uniform separation of adipocytes over the incubation period of 60 minutes. Collagenase (type II) was therefore added to each of sample preparations in the following concentrations: Inguinal and popliteal, 1.3 mg ml⁻¹, mesenteric and omental, 1.5 mg ml⁻¹, epididymal, 1.0 mg ml⁻¹.

The samples were incubated at 37°C in a shaking water bath (1 Hz) for up to 60 minutes. At the end of the incubation period the vials were given a quick, but not too vigorous, shake to liberate the adipocytes from the tissue fragments. The adipocyte suspensions were then passed through a 230 µm screen cup (Filter no. 60 mesh, Sigma) into a petri dish to remove undigested tissue. The adipocytes were resuspended in 10 ml of wash buffer and centrifuged for 2 minutes at 1500 g, in a Beckman centrifuge, model TJ-6. This procedure was repeated 3 times to wash the adipocytes and remove any stromal-vascular cells present. The washed adipocytes were resuspended in 10 ml of wash buffer and kept at 37°C in a waterbath prior to glutamine uptake assay. An aliquot of 10 µl of adipocyte suspension was taken from each adipocyte preparation for cell counting. Protein was estimated according to the method of Bradford (1976) to express data per mg protein. Cell viability was checked using the trypan blue exclusion method.

3.2.6. A preliminary study of glutamine uptake into isolated epididymal adipocytes

The preliminary investigation of glutamine uptake over time was performed to determine whether the method used in this study was suitable for measuring glutamine uptake in isolated adipocytes. In addition, incubation of adipocytes over the time course was used to

establish by which time point a steady-state intracellular concentration of glutamine was achieved.

Epididymal adipocytes were prepared from 4 guinea-pigs and glutamine uptake was measured using the following procedure:

The cell suspension was gently mixed in the wash buffer by swirling the tubes to ensure an even distribution of adipocytes. Aliquots (200 μ l) of the cell suspension were added to 1.5 ml Eppendorf tubes containing 800 μ l of preincubation buffer previously warmed to 37°C. The adipocytes were preincubated for 30 minutes at 37°C under a stream of O₂, 95%/CO₂, 5%.

Following preincubation, the preincubation buffer was aspirated off using a 2 ml plastic syringe. Incubation media (800 μ l) was added to the adipocytes which were incubated for 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0 and 10 minutes. After the appropriate incubation period, the reaction was terminated by the addition of 100 μ l ice cold solution of ouabain (10 μ M). The samples were then placed immediately on ice. Controls, in duplicate, included 200 μ l of cell suspension treated in the same way as the rest of the incubations but set on ice throughout the incubation period to account for radioactivity trapped on the surface of, or between, the cells. The values of radioactivity measured in these control samples were deducted from all subsequent values obtained with incubated adipocytes.

The adipocytes were separated from the incubation buffer through an inert oil, di-"isononyl" phthalate (specific gravity 0.98) by the method of Gliemann *et al.* (1972). For this procedure, 200 μ l of di-"isononyl" phthalate was added to the cell suspension which were then centrifuged for 2 minutes in an Eppendorf minifuge at 1500 *g*. The cells formed a layer on the top of the oil, which has a lower density than the incubation buffer and higher density than the adipocytes. The incubation buffer was separated from the oil and cells by piercing the bottom of the tubes, the buffer was allowed to drain leaving the oil and cells at the base of the Eppendorf tubes. The bottom section of the tubes containing the oil and cells were cut with scissors and transferred into 13 ml plastic scintillation vials. Eight ml of scintillation fluid (Optiphase HIsafe 3, Pharmacia) was dispensed into each

vial. The samples were left for at least 2 hours to ensure that cells were fully dissolved and vortexed vigorously immediately prior to counting to ensure an even incorporation of dissolved cells and oil into the scintillation fluid. L-[$^{14}\text{C}(\text{U})$] glutamine uptake was estimated by scintillation counting (3 minutes per sample, in a Beckman 1701 scintillation counter).

The following controls were included in these preliminary trials of glutamine uptake into adipocytes:

1. 100 μl of oil was counted after separation from the medium to ensure that the label had not been incorporated into the oil.
2. To ensure that counts within the incubation buffer remained similar (as excess label represents an inbuilt marker of assay stability), 100 μl of infranatant (buffer) was counted over a time course of between 30 seconds and 3 minutes incubation.

3.3. Experimental procedures

3.3.1. The effect of insulin, dexamethasone and isoprenaline on glutamine uptake into isolated adipocytes prepared from five adipose sites

Isolated adipocytes prepared from adipose sites as described above (Section 3.2.3) were preincubated for 30 minutes along with control samples which were set on ice. The effect of insulin, (concentration range 0.1 to 1000 $\mu\text{U ml}^{-1}$), isoprenaline (concentration range 1 to 100 μM), dexamethasone (concentration range 100 to 2500 nM) and dexamethasone (2500 nM) with insulin (10 $\mu\text{U ml}^{-1}$) on glutamine uptake into isolated adipocytes was measured using the following procedure:

Aliquots of 200 μl cell suspension was added in duplicate to the incubation buffer, supplemented with the appropriate concentrations of bovine insulin, isoprenaline, dexamethasone or insulin plus dexamethasone. The cells were incubated at 37°C for 5 minutes, as glutamine uptake had attained a stable level by this point in the above preliminary time course experiments (Figure 3.1). The reaction was terminated, and the cells were separated from the incubation buffer as described above (Section 3.2.2.).

3.3.2. Measurement of serum cortisol levels in fasted and fed guinea-pigs

Serum cortisol levels were measured by radioimmunoassay using a Gamma-BCT cortisol kit supplied by Immuno Diagnostic Systems, Bolden, Tyne and Wear, England.

Guinea-pigs were either fasted overnight for 18 hours or fasted overnight then given access to food for 30 minutes before serum collection. During this procedure to collect the serum, the animals were anaesthetised with 3% pentobarbital as described in Section 3.2.1. and blood was extracted by an experienced animal technician using cardiac puncture. The blood was placed in 13 ml non-heparinised plastic Sarstedt tubes and left at 4°C for 2 hours then centrifuged at 1000 g for 5 minutes. Serum was removed and stored at -20 °C. Before measurement the serum was diluted 1:2 and 1:4 in PBS.

Serum cortisol concentrations were estimated using a standard curve and values were expressed as nmol l⁻¹.

3.3.3. Presentation of data and statistical analysis

Net uptake of L-[¹⁴C(U)] glutamine in 4 separate time course experiments, was calculated by subtracting the mean radioactive counts per minute obtained from the control samples (Section 3.2.7) from counts obtained from the time course samples. The net mean counts for the samples, were then expressed as nmol glutamine uptake mg⁻¹ protein (L-[¹⁴C(U)] glutamine plus unlabelled glutamine). Differences in glutamine uptake were tested for significance between control and treated (insulin, dexamethasone or isoprenaline) samples by repeated-measures ANOVA.

3.4. Results

3.4.1. Preliminary study of glutamine uptake in isolated epididymal adipocytes over a time course of 10 minutes

Glutamine uptake into isolated epididymal adipocytes was rapid with time, incorporation reaching a maximum of 0.13 nmol min⁻¹ mg⁻¹ protein after 5 minutes of incubation time (Figure 1.). This value of uptake was maintained at a steady-state when measured after 10

minutes of incubation. From 0 to 0.5 minutes there was a lower rate of glutamine uptake than from 0.5 to 2 minutes, possibly indicating two separate transport mechanisms.

On the basis of this preliminary study, an incubation time of 5 minutes was chosen for the adipocytes in the subsequent hormone studies.

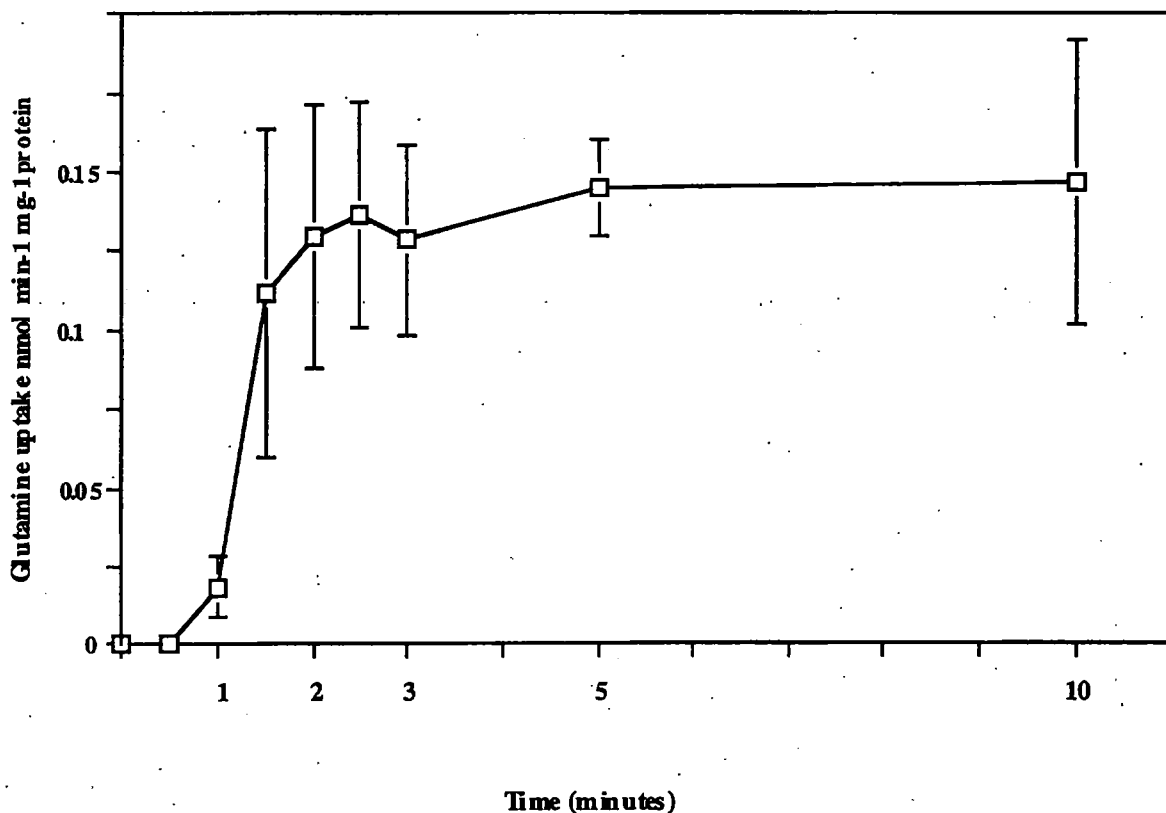


Figure 3.1. Glutamine uptake over a time course of 10 minutes. Expressed as nmol min⁻¹ glutamine incorporated mg⁻¹ protein (mean and SE) in isolated adipocytes, (n = 4).

3.4.2. The effect of insulin on glutamine uptake in isolated adipocytes from different adipose sites

Glutamine uptake into adipocytes isolated from all sites was relatively low in the absence of insulin, but there was a dose-dependent increase in the presence of insulin (Figures 3.2 and 3.3). There were some site-specific differences in the capacity of insulin to modulate glutamine uptake. In adipocytes prepared from the inguinal site, glutamine uptake

increased significantly ($P < 0.001$) with $1.0 \mu\text{U ml}^{-1}$ insulin compared to the control values and a maximum uptake was attained with an insulin concentration of $10 \mu\text{U ml}^{-1}$ (Figure 3.2) which is within the physiological concentration range. Thereafter, glutamine uptake in inguinal adipocytes was not further increased over the range of insulin concentrations. In adipocytes prepared from the omental and epididymal sites, mean uptake glutamine increased significantly ($p < 0.01$) with an insulin concentration of $1.0 \mu\text{U ml}^{-1}$ and maximum was attained with a higher insulin concentration of between 10 and $100 \mu\text{U ml}^{-1}$ (Figures 3.2 and 3.3). In the popliteal and mesenteric sites, glutamine uptake was significantly increased compared to that of the control values with an insulin concentration of $1.0 \mu\text{U ml}^{-1}$ ($p < 0.01$) and adipocytes from these sites showed the lowest maximal response of glutamine uptake (0.13 and $0.19 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein mesenteric and popliteal respectively, with insulin $1000 \mu\text{U ml}^{-1}$, Figures 3.2 and 3.3). Adipocytes prepared from the omental and inguinal sites demonstrated the greatest response to insulin and highest maximum rate of glutamine uptake of 0.3 and $0.27 \text{ nmol min}^{-1} \text{ mg}^{-1}$ respectively (Figures 3.2 and 3.3).

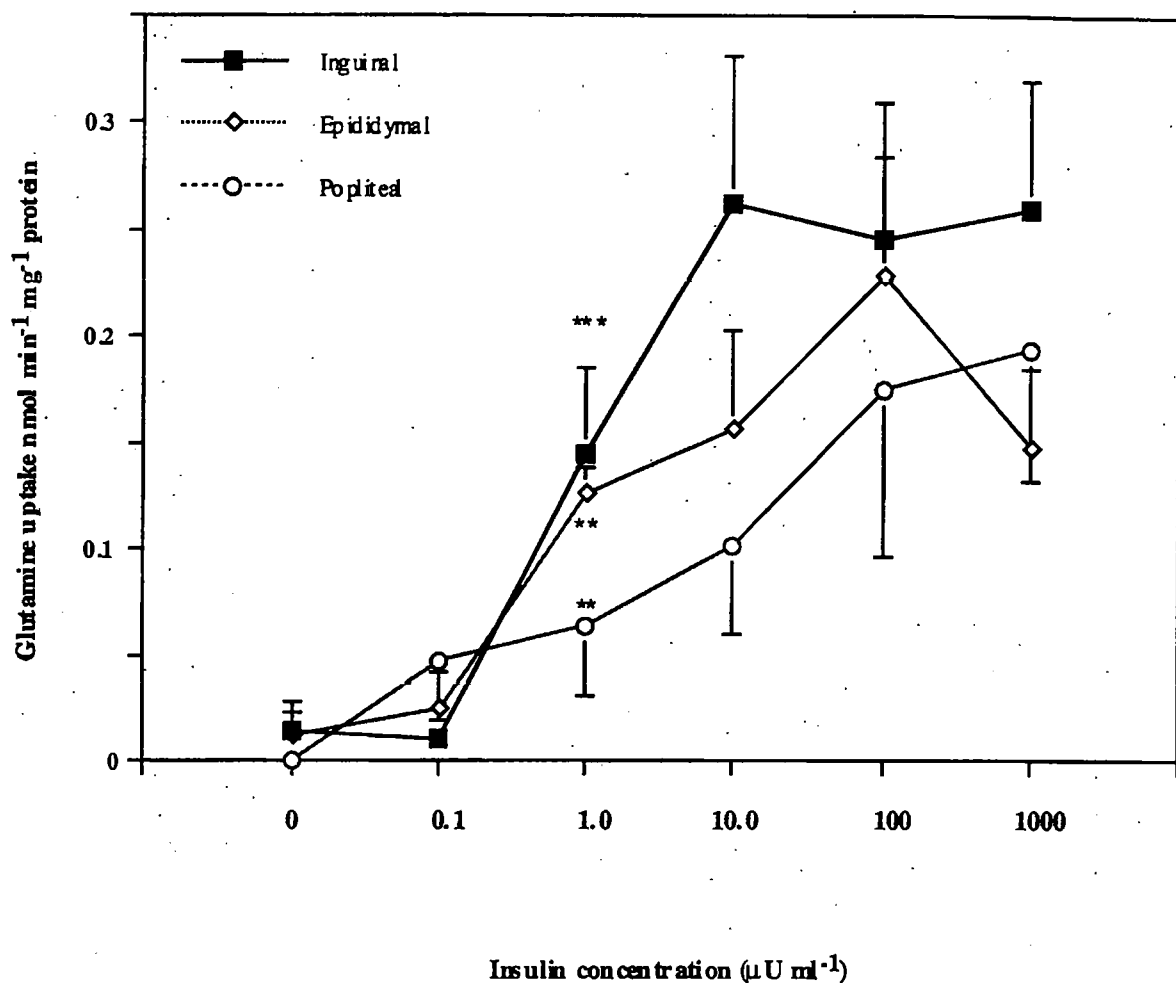


Figure 3.2. Glutamine uptake with different concentrations of insulin. Expressed as nmol glutamine incorporated $\text{min}^{-1} \text{mg}^{-1}$ protein, in isolated adipocytes prepared from inguinal, epididymal and popliteal sites ($n=4$). Statistical significance compared to control values (no insulin), ** $p<0.01$, *** $p<0.001$.

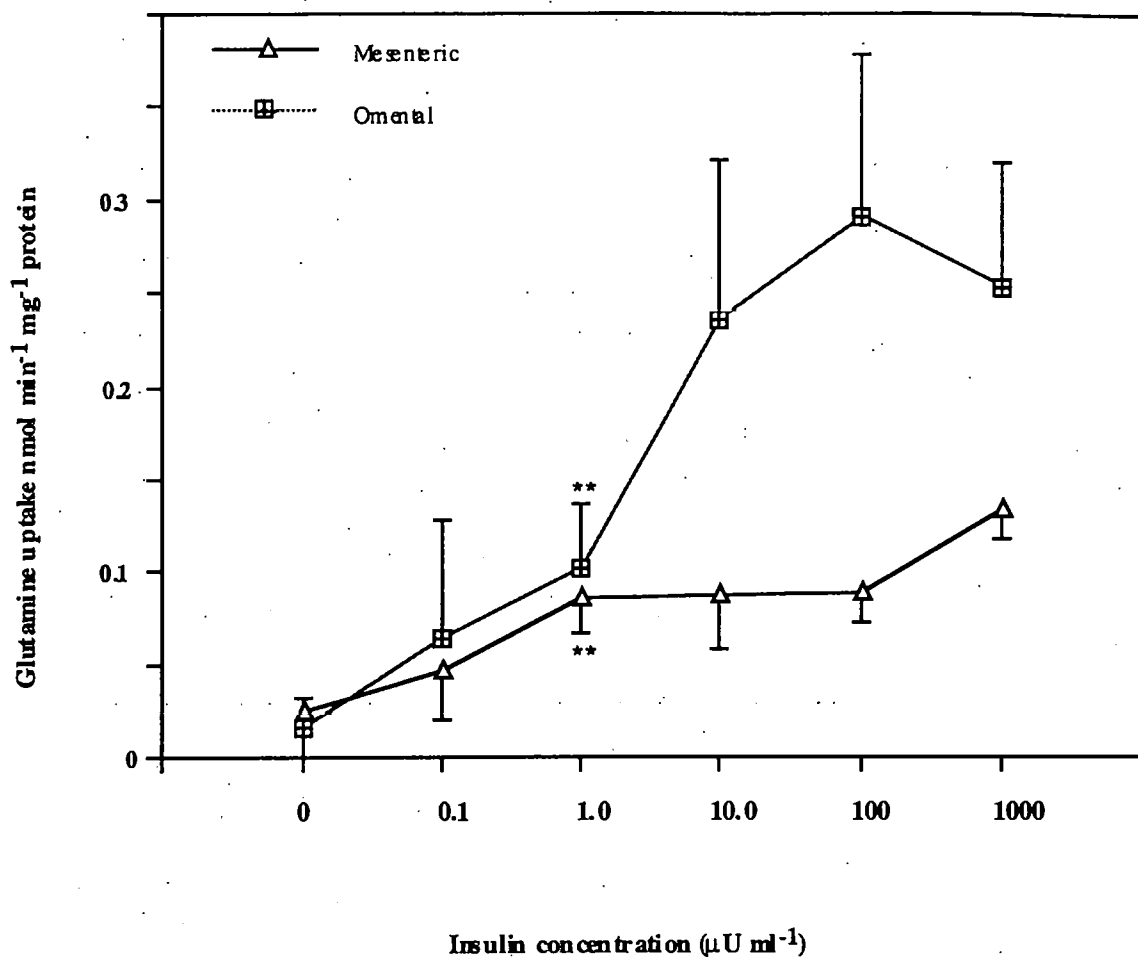


Figure 3.3. Glutamine uptake with different concentrations of insulin. Expressed as nmol glutamine incorporated min⁻¹ mg⁻¹ protein, in isolated adipocytes prepared from mesenteric and omental sites (n=4). Statistical significance compared to control values (no insulin), ** p<0.01, ***p<0.001.

3.4.3. The effect of dexamethasone and insulin with dexamethasone on glutamine uptake in isolated adipocytes from different adipose sites

A preliminary trial incubation with dexamethasone (concentration range of between 100 and 2500 nM) had no significant effect on glutamine uptake in adipocytes prepared from any of the sites studied (results for dexamethasone, 2500 nM shown in Figure 3.4).

In agreement with the previous study, insulin ($100 \mu\text{U ml}^{-1}$), significantly stimulated glutamine uptake into adipocytes from all depots (Figure 3.4). Dexamethasone ($2.5 \mu\text{M}$) did not significantly potentiate the effect of insulin ($100 \mu\text{U ml}^{-1}$) on glutamine uptake in adipocytes prepared from any of the sites studied.

3.4.4. The effect of isoprenaline on glutamine uptake in isolated adipocytes from different adipose sites

There was a significant decrease in glutamine uptake compared with basal values in adipocytes prepared from the inguinal site incubated with isoprenaline (100-1000 nM), (Figure 3.5). Glutamine uptake was also decreased in adipocytes prepared from the mesenteric, omental, and epididymal sites compared with basal values, when incubated with a high dose of isoprenaline (1000 nM), (Figures 3.5 and 3.6). No significant change in glutamine uptake with any concentration of isoprenaline studied was observed in adipocytes prepared from the popliteal site (Figure 3.5).

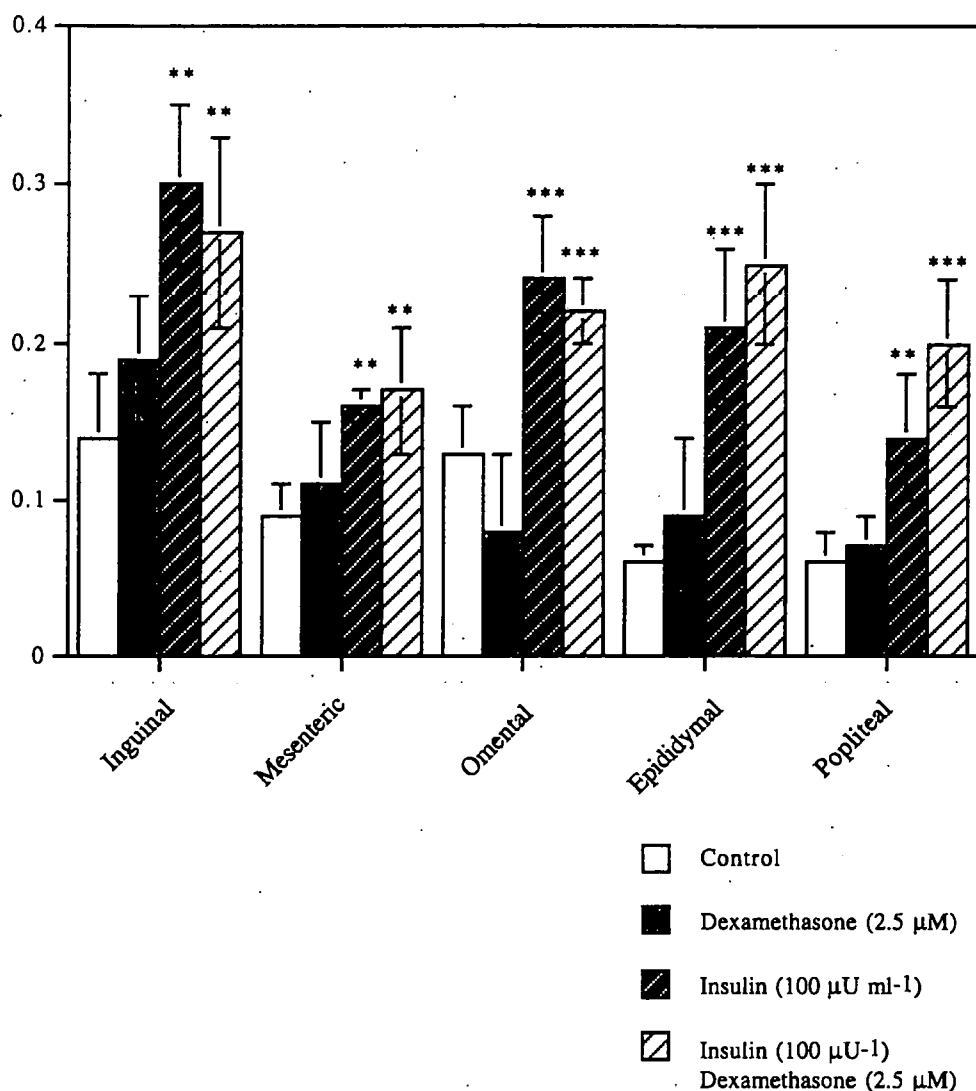
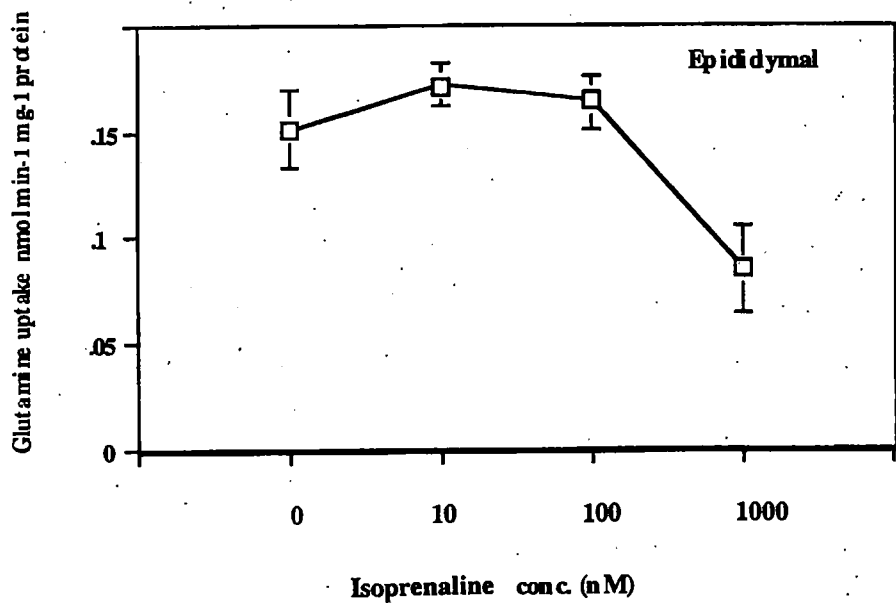
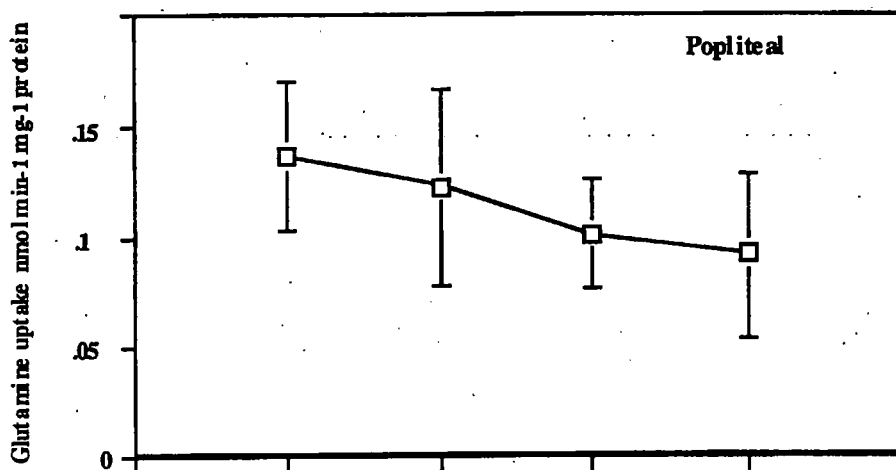
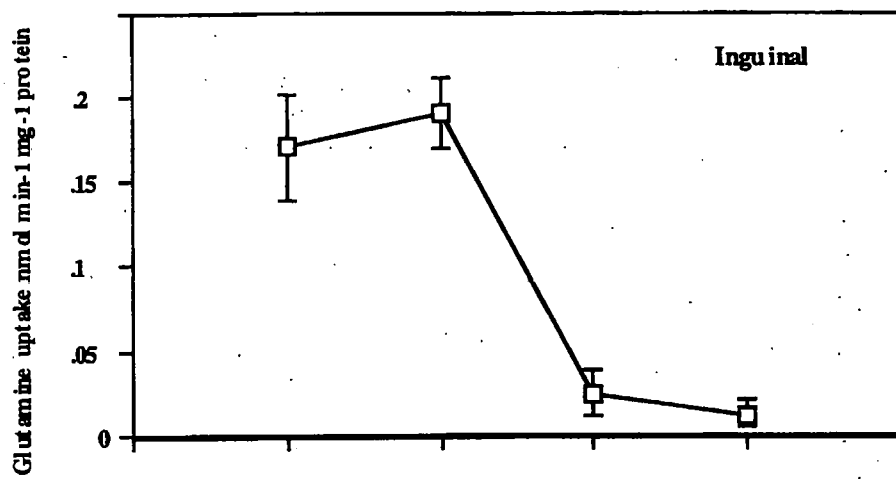


Figure 3.4. Glutamine uptake with insulin (100 μ U ml⁻¹), dexamethasone (2.5 μ M) and insulin with dexamethasone. Expressed as nmol glutamine incorporated min⁻¹ mg⁻¹ protein, in adipocytes isolated from inguinal, mesenteric, omental epididymal and popliteal sites (n=4). Statistical significance compared to control values (no insulin), ** p<0.01, ***p<0.001.



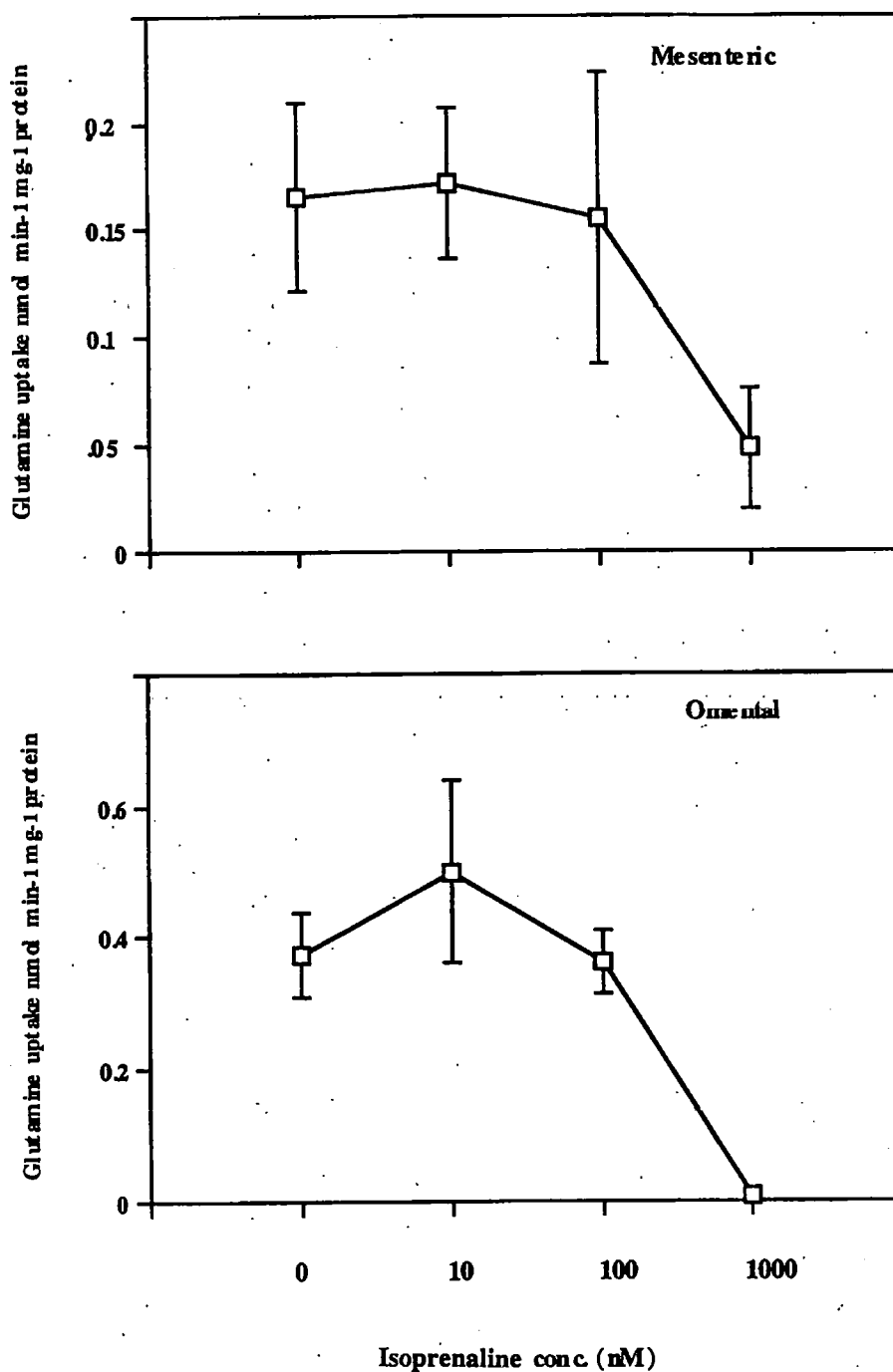


Figure 3.6. Glutamine uptake with different concentrations of isoprenaline. Expressed as nmol glutamine incorporated min⁻¹ mg⁻¹ protein, in isolated adipocytes prepared from mesenteric and omental sites (n=4).

Table 3.4.5. Serum cortisol levels from fasted and fed guinea-pigs (n=4)

Condition:	Cortisol nmol l⁻¹
Fasted overnight	3089.7 ± 100.1
30 min. after a meal	1939 ± 456.4**

Serum cortisol levels were significantly lower in the fed animals ** denotes $P < 0.01$.

3.5 Discussion

The aims of this study were:

1. To measure the effect of insulin, dexamethasone and isoprenaline on glutamine uptake in adipocytes isolated from different adipose depots and determine whether there were site-specific differences in the pattern of glutamine uptake.
2. To compare the effects of insulin and the hormone analogues on glutamine uptake with the changes in glutaminase activity after feeding, fasting and exercise and to determine whether there were any similarities between the site-specific pattern of glutamine uptake with different agonists and the change in glutaminase activity of the same tissues detailed in Chapter 2.

3.5.1. Investigation of glutamine uptake into isolated adipocytes over time

The results shown in Fig. 3.1 demonstrate that glutamine is incorporated into isolated adipocytes over a time course of 10 minutes. The sigmoidal pattern of glutamine incorporation into adipocytes, with a V_{\max} of approximately $0.13 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein, indicates that this process is probably facilitated by active cell membrane transport rather than by passive diffusion alone.

3.5.2. The effect of insulin on glutamine uptake into isolated adipocytes

The data demonstrate a dose-dependent uptake of glutamine in response to insulin (Figures 3.2 and 3.3). Information on the effect of insulin on glutamine uptake in isolated adipocytes is limited. However, a study by Kowalchuk *et al.* (1988) reported that the rate of glutamine utilisation was increased compared with basal values in rat epididymal adipocytes incubated with a very high physiological concentration of insulin (10 mU ml^{-1}) and glucose (1 mM). The results from the present study (Section 3.4.2) show that there was a detectable stimulatory effect on glutamine uptake at the lower end of the physiological range of insulin concentration ($1.0 \text{ } \mu\text{U ml}^{-1}$) by adipocytes isolated from inguinal, epididymal and popliteal adipose sites (Figure 3.2) and by those isolated from the omental site (Figure 3.3). In addition there was a much larger effect of insulin ($10 \text{ } \mu\text{U ml}^{-1}$) in all sites except mesenteric. The mechanism by which glutamine uptake is enhanced by insulin in adipocytes is not known. There is however, some information on the effect of insulin on glutamine transport into skeletal muscle. Insulin has been shown to stimulate glutamine uptake in perfused rat hindquarters and in cultured rat skeletal myocytes (Hundal *et al.* 1987 and Tadros *et al.* 1993). It has been proposed that stimulation by insulin of glutamine transport occurs via an increase in the capacity of the "N^m" system which serves to increase V_{max} without affecting K_m (Hundal *et al.* 1987 and Tadros *et al.* 1993). The mechanism of glutamine uptake in muscle may share some similarities with that of adipose tissue since both tissues are of the same embryological origin. In addition, it is thought that the metabolism of amino acids in muscle and adipose tissue share some similarities in the catabolism of the BCAAs leucine, isoleucine and valine and in the synthesis and release of valine (Tischler and Goldberg 1980; Snell and Duff 1977).

The action of insulin on glutamine uptake into muscle, and into certain adipose depots (superficial and intra-abdominal) demonstrated by this study, would therefore direct the post-prandial flow of this amino acid into these tissues. Furthermore, the increase in glutaminase activity measured in adipose tissue extracts after feeding (Chapter

2), combined with an increase in the intra-cellular availability of glutamine, indicate that insulin may serve to stimulate glutamine utilisation in adipose tissue post-prandially.

In the present study, the fate of glutamine in the adipocyte was not studied, but in the previously mentioned study by Kowalchuk *et al.* (1988) it was reported that in rat epididymal isolated adipocytes, the glutamine carbon is incorporated into triacylglycerol when insulin (10 mU ml^{-1}) and glucose (1 mM) are included in the incubation medium. The data from the glutaminase study after feeding, shown in Chapter 2, along with data from this Chapter suggest that the omental and inguinal depots have a significant capacity to utilise glutamine, possibly as a substrate for triacylglycerol synthesis in the early post-prandial period.

3.5.3 Site-specific differences in glutamine uptake with insulin into isolated adipocytes compared with glutaminase activity in adipose tissue extracts

There were site-specific differences in the response to different concentrations of insulin and in the maximal rate of glutamine uptake. The increase was greatest in adipocytes prepared from the omental (far from spleen) and inguinal depots. Furthermore, half maximal stimulation of uptake in adipocytes from these sites was achieved with differing concentrations of insulin suggesting both an effect on K_m and V_{max} . In adipocytes isolated from the inguinal, epididymal and omental sites glutamine uptake was increased at physiological insulin concentration of between $10 - 100 \mu\text{U ml}^{-1}$ to a greater extent than those isolated from the popliteal and mesenteric sites. Furthermore, the response of mesenteric adipocytes was low, requiring an insulin concentration outside the physiological range of $1000 \mu\text{U ml}^{-1}$ for maximal glutamine uptake. The molecular structure of guinea-pig insulin differs from that of insulin from other domestic and laboratory mammals (Blundell and Humbel 1980). As adipocytes from some depots responded to bovine insulin by an increase in glutamine uptake, it is likely that the low response to insulin in adipocytes prepared from the popliteal and mesenteric sites were real and were not due to the use of bovine insulin. In the presence of natural guinea-pig

insulin, the site-specific differences in adipocytes would probably persist and the differences in the magnitude of the response between the sites may be enhanced.

Site-specific differences in glutaminase activity were also measured in adipose tissue extracts after feeding (Chapter 2). At 30 minutes after feeding, adipose tissue from the inguinal site had the highest value of glutaminase activity compared to that of all other sites (except omental, adjacent to the spleen, Chapter 2, Section 2.4.8). In this study, glutamine uptake into inguinal adipocytes was significantly increased in the presence of insulin ($10 \mu\text{U ml}^{-1}$). The response to insulin measured in inguinal adipocytes together with high value of glutaminase activity observed after feeding in adipose tissue extracts from the same site suggest that this large depot has a significant capacity to take up and utilise glutamine post-prandially.

There were also increases in glutaminase activity after feeding in adipose tissue extracts from other sites studied. However, the results of this study show that glutamine uptake in adipocytes isolated from the popliteal site responded only weakly to insulin. Increases in glutaminase activity after feeding were lowest in adipose tissue extracts from this site (Chapter 2). The small change in glutaminase activity after feeding (Chapter 2, Table 2.3) together with the relatively low response of adipocytes to insulin-stimulated glutamine uptake (Figure 3.2) indicates that the popliteal depot does not significantly contribute to the extraction and utilisation of glutamine post-prandially.

Glutaminase activity was increased in mesenteric adipose tissue extracts after feeding (Chapter 2, Table 2.3). However, adipocytes isolated from this site were the least affected by insulin in this study, in that uptake was only significantly increased with physiologically high concentration of insulin ($1000 \mu\text{U ml}^{-1}$). The low response to insulin in mesenteric adipocytes may be a result of its close proximity to, and shared blood supply, with the intestine. Glutamine absorbed from the diet could pass freely into the portal circulation without being sequestered by mesenteric adipose tissue. The increase in glutaminase observed immediately after feeding (Chapter 2) however, suggests that this site maintains the capacity to utilise glutamine when intracellular concentrations of this amino acid are increased.

The site-specific differences in insulin-mediated glutamine uptake into adipocytes together with site-specific differences in glutaminase activity after feeding suggest that the contribution of insulin to the regulation of utilisation of this amino acid in adipose tissue differs between depots. The lower capacity of popliteal and mesenteric adipose tissue to extract and utilise significant quantities of glutamine after feeding compared to other sites may be associated with their distinct anatomical relationship with muscle and the intestine respectively.

The popliteal depot is closely associated with, and shares a blood supply with, skeletal muscle which, *in vitro* consumes glutamine at a high rate (Partridge and Casanello-Ertl 1979). In addition, a high activity of glutaminase has been detected in both human and rat skeletal muscle mitochondria (Swierczyński *et al.* 1993). The relative contribution of muscle and adipose tissue to the disposal of circulating glutamine has yet to be established. However, as glutamine uptake into muscle has been shown to be enhanced by insulin, it is not inconceivable that post-prandially, glutamine uptake in adipose tissue may also be enhanced by insulin and contribute to the removal of this amino acid from the circulation. The close proximity of the popliteal depot to skeletal muscle (which may also consume glutamine) coupled with its small mass relative to other adipose sites may therefore be determining factors in the overall capacity of the popliteal depot to contribute to whole-body glutamine disposal post-prandially.

Mesenteric adipose tissue is attached to, and shares a blood supply with, the small intestine. The low rate of insulin-stimulated glutamine uptake in this site would therefore allow this amino acid to pass relatively unimpeded through the portal vein to the liver and peripheral circulation.

The site-specific differences in both glutaminase activity after feeding and insulin stimulated glutamine uptake would serve to direct the flow of this amino acid in the post-prandial state to the inguinal, omental (far from spleen) and epididymal adipose depots but away from the popliteal and mesenteric depots.

3.5.4 The effect of dexamethasone and insulin with dexamethasone on glutamine uptake into isolated adipocytes compared with glutaminase activity in fasted and fed guinea-pigs

Guinea-pigs are known to have high circulating levels of cortisol in comparison with other mammals that have been studied (Smith *et al.* 1987). In this study, measurements of serum cortisol levels were higher after an overnight fast than at 30 minutes after feeding (Section 3.4.5). Contrary to the stimulatory effect of dexamethasone on glutamine utilisation in the small intestine of rats (Souba 1993; Ardawi *et al.* 1988), the data from the present study show no demonstrable effect of this hormone analogue with or without insulin, on glutamine uptake in adipocytes isolated from the five sites included in this study. Furthermore, glutaminase activity in adipose tissue from the fasted animals was reduced compared to that measured at 30 minutes after a meal. Elevated fasting plasma levels of cortisol (Section 3.4.5) along with relatively low glutaminase activity in adipose tissue may therefore serve to reduce the flow of glutamine into adipose tissue in the fasted state and direct it towards utilising tissues such as the liver for gluconeogenesis or to lymphoid tissue.

3.5.5. The effect of isoprenaline on glutamine uptake into isolated adipocytes compared with changes in glutaminase activity following acute exercise

The data from this study demonstrate a significant reduction in glutamine uptake with isoprenaline in adipocytes isolated from the inguinal, epididymal, mesenteric and omental sites. Adipocytes isolated from the inguinal site were highly responsive to isoprenaline at a concentration of 10 nM, whereas a much greater concentration of 1000 nM was required for a significant reduction in glutamine uptake in the other sites studied. No significant change in adipocytes isolated from the popliteal site could be demonstrated in glutamine uptake with increasing concentrations of isoprenaline.

Isoprenaline mimics the effects of the catecholamines and was used in this study as it has been shown to be a potent lipolytic agent in human and guinea-pig adipocytes via stimulation of the β_1 -adrenoceptor (Lafontan and Berlan 1993). Stimulation of β -adrenergic receptors serves to activate adenylyl cyclase, leading to an increase in cAMP production and subsequent activation of protein kinase A, which in turn phosphorylates hormone-sensitive lipase thus initiating lipolysis. Other short-term metabolic effects of β -adrenergic activation in adipocytes are associated with stimulation of glycogenolysis, and inhibition of glucose transport.

Amino acid transport and metabolism in isolated adipocytes has been shown to be inhibited by agents which are known to raise intracellular levels of cAMP such as adrenocorticotrophic hormone (ACTH), adrenaline, and theophylline (Minemura *et al.* 1970; Touabi and Jeanrenaud 1969). The results from this study demonstrate that the uptake of glutamine into isolated adipocytes is also inhibited by isoprenaline, an agent known to increase intracellular cAMP levels. The process by which β -adrenergic stimulation inhibits glutamine uptake into adipocytes has not been investigated. Stimulation of lipolysis and subsequent accumulation of intracellular free fatty acids before they are released together with a fall in ATP levels may play a role in the reduction of glutamine uptake into adipocytes. Furthermore, alterations in cell membrane transport which favour the release of free fatty acids from the adipocyte may also alter the capacity for glutamine uptake.

A reduction in glutaminase activity was also measured in adipose tissue extracts following exercise in the inguinal, omental, mesenteric, epididymal and popliteal sites (Chapter 2). Activation of the sympathetic nervous system leading to stimulation of catecholamine secretion associated with exercise and stress could therefore serve to both reduce glutamine uptake and its subsequent hydrolysis, thus diverting the flow of glutamine away from adipose tissue perhaps towards muscle or liver.

Conclusions

The study of glutaminase activity in adipose tissue extracts after feeding, fasting or exercise described in Chapter 2 indicates that enzyme activity may in part be altered by the effects of different hormones and neural transmitters present in the circulation, such as insulin, glucocorticoids and catecholamines. Furthermore, there were site-specific differences in glutaminase activity after feeding, fasting and exercise.

Insulin and isoprenaline in isolated adipocytes have inverse effects on glutamine uptake. Insulin enhances both glutamine uptake and probably glutaminase activity in certain adipose tissue depots which would lead to a post-prandial increase in glutamine removal from the circulation and its subsequent hydrolysis. Adipose tissue, through the effects of insulin may therefore play an anabolic role post-prandially in which the product of glutamine hydrolysis, namely glutamate, may serve as a substrate for lipogenesis or for further amino acid synthesis.

Conversely, the reduction in glutamine uptake with isoprenaline into isolated adipocytes demonstrated in this study in conjunction with the fall in glutaminase activity with exercise suggests that stimulation of the sympathetic nervous system would serve to limit the metabolism of glutamine in adipose tissue under catabolic conditions. These findings support the hypothesis that hormones associated with feeding and exercise play a part in regulating both the uptake and subsequent metabolism of glutamine into specific adipose tissue depots.

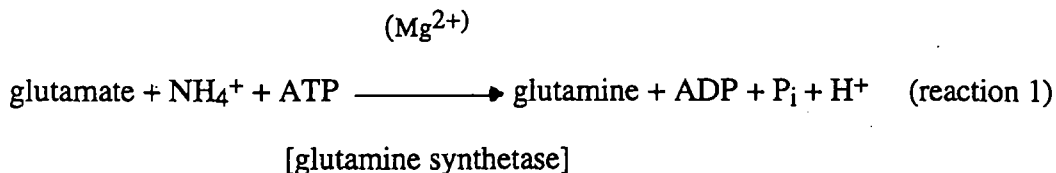
Furthermore site-specific differences in glutamine uptake and glutaminase activity in response to changing physiological conditions demonstrate that adipocytes have certain properties which may favour local interaction with tissues and organs and determine differing contributions to whole-body glutamine homeostasis.

Chapter 4

**A study of glutamine synthetase
activity following feeding, fasting
and exercise in adipose tissue from
different depots**

4.1. Introduction

Glutamine synthetase (E.C. 6.3.1.2) catalyses the formation of glutamine from glutamate and ammonia. This amidation is coupled to the cleavage of ATP to ADP. A divalent cation such as Mg^{2+} or Mn^{2+} is also required (reaction 1).



Through reaction 1. glutamine synthetase incorporates free ammonia into glutamine thereby assimilating this toxic compound into non-toxic metabolites in tissues.

Glutamine synthetase was first isolated from sheep brain (Pamijans, *et al.* 1962), and has since been identified in many other tissues including liver (Meister 1984), muscle (King *et al.* 1983), lymph nodes (Ardawi and Newsholme 1984b), intestine (Ardawi *et al.* 1988) lung (Ardawi 1990) liver, kidney (Watford 1991), white adipose tissue (Miller 1975 and Kowalski and Watford 1994b) and brown adipose tissue (López-Soriano and Alemany 1987).

Glutamine plays a range of metabolic functions in different tissues (discussed in Chapter 1, Section 1.2) so efficient regulation of this enzyme is essential. Alterations in glutamine synthetase activity may play a key role in both the maintenance of glutamine supply within tissues and in the provision of circulating glutamine for whole body glutamine metabolism. Increased utilisation of glutamine by different tissues must be balanced either by increased production or decreased utilisation at other sites within the body. Therefore, it is essential to identify tissues that synthesise and release glutamine and understand what factors influence glutamine synthetase activity in these tissues to assess their quantitative contribution to the maintenance of whole-body glutamine homeostasis.

Skeletal muscle is thought to contribute significantly to glutamine synthesis and release in the mammalian body (Rudermann and Berger 1974). However, studies in several species have shown that glutamine uptake by visceral organs exceeds glutamine release by skeletal muscle (Pozefsky *et al.* 1969, Felig 1975, Welbourne 1987 and

Heitmann and Bergman 1978). Other tissues which contribute to circulating glutamine levels have yet to be fully described, and in the rat may include the lung (Souba *et al.* 1990). A recently identified site of glutamine synthesis and production is adipose tissue (Frayn *et al.* 1991, Kowalski and Watford 1994a). Glutamine synthetase activity in adipose tissue expressed per mg of protein has been shown to be greater than that measured in skeletal muscle (Miller 1975 and Opara 1993), but little is known about the regulation of this enzyme in adipose tissue *in vivo*.

Glutamine synthetase activity in cultured murine 3T3-L1 pre-adipocytes is known to be modulated by insulin, hydrocortisone and dibutyryl cyclic AMP (dbcAMP) (Miller and Burns 1985). Glutamine synthetase activity in this cell line increases 100 fold during differentiation to adipocytes (Miller *et al.* 1983). In differentiating and in fully differentiated 3T3-L1 cells, glutamine synthetase mRNA, glutamine synthetase protein and enzyme activity are increased when hydrocortisone is added to the incubation medium whereas insulin has little effect upon, or decreases, glutamine synthetase activity, protein and mRNA (Miller *et al.* 1978; Miller *et al.* 1983; Saini *et al.* 1990). The addition of dbcAMP to the incubation medium results in a reduction in glutamine synthetase activity and its protein, coupled to an increase in mRNA degradation (Bhandari *et al.* 1988; Saini *et al.* 1990).

Site-specific differences between adipose depots in the activity of glutamine synthetase have also been reported in fed and fasted rats (Kowalski and Watford 1994b). However, no extensive studies have been carried out on the activity of this enzyme in the main adipose depots of laboratory rodents under controlled conditions of fasting, feeding or exercise over a time course. Such information may help to elucidate the role of adipose tissue in maintaining whole body glutamine homeostasis with different physiological states.

4.1.1 Hypothesis:

There are site-specific differences in the capacity of adipose tissue to synthesise glutamine through the action of glutamine synthetase. Site-specific differences in glutamine synthetase activity between adipose tissue depots with altering physiological status, play a part in regulating the supply of this amino acid for local and/or whole-body glutamine metabolism.

4.1.2 Aims

The aims of this study were to investigate the effects of different physiological conditions on glutamine synthetase activity in different adipose depots *ex vivo*. Determination of physiological factors that affect glutamine synthetase activity in different adipose depots may help to elucidate the contribution of this tissue to the regulation of whole-body and local glutamine supply.

4.2. Materials and Methods

4.2.1 Chemicals

Chemicals and enzymes detailed in Appendix II.

4.2.2. Dissection procedure

The guinea-pigs were sacrificed between 0930 and 1030 h with an intra-peritoneal overdose injection of 3% pentobarbital dissolved in phosphate buffered saline. 0.5 g samples of adipose tissue from 11 different adipose sites were dissected onto ice and weighed as previously described in Chapter 2. Section 2.2.3.

4.2.3. Preparation of homogenates

The dissection took a maximum of 20 minutes. The adipose tissue from each site was chopped with scissors and placed in 13 ml Sarstedt test tubes set on ice containing 6 x volume of extraction buffer; Tris 50 mM, EDTA 2 mM, pH 7.9. The samples were kept

on ice and homogenised on ice for 30 seconds (Polytron homogeniser, setting 4). The homogenate was centrifuged at 200 g for 15 minutes at 0°C to remove debris. The supernatant between a layer of lipid and a protein pellet was used for the enzyme assay.

The pellet was retained for protein determination using a modified method of Bradford (1976) see Appendix I.

4.2.4. Glutamine synthetase assay

Glutamine synthetase activity was measured using a radiochemical assay modified from that of King *et al.* (1983). This assay uses a phosphocreatine/creatine kinase ATP regenerating system which minimizes inhibition of glutamine synthetase by ADP (Meister 1984).

The reaction was initiated by the addition of duplicate samples of 50 µl adipose tissue homogenate supernatant to 360 µl of assay medium. Blanks consisted of 50 µl of extraction buffer and 360 µl reaction medium. The assay medium was made up in 10 ml of de-ionised water immediately prior to the assay and consisted of:

Chemical	Concentration
Imidazole hydrochloride	50 mM
Magnesium chloride	20 mM
Ammonium chloride	20 mM
Sodium glutamate	20 mM
ATP	15 mM
Creatine phosphate	10 mM
Creatine kinase	1.2 units

The pH of the assay medium was adjusted to 7.6 at room temperature and 250 µl glutamic acid, L-[¹⁴C(U)], (50 µCi ml⁻¹) was added.

The samples and blanks were incubated at 37°C for 30 minutes in a waterbath and the reaction terminated by the addition of 100 µl ice cold perchloric acid (2%). The incubation samples and blanks were centrifuged in an Eppendorf minifuge at 10,000 g for 2 minutes, and neutralised using 8-10 µl of KOH 2 M, TRA 0.5 M solution and 10 µl of universal indicator. Samples and blanks were centrifuged again (10,000 g for 2 minutes) to remove the precipitate formed during neutralisation.

[¹⁴C]-glutamine formed in the reaction medium was separated from its precursor [¹⁴C]-glutamate by ion-exchange chromatography, using a method modified from that of Pishak and Phillips (1979); 360 µl of neutralised samples were added to 2.5 ml syringes, plugged with Whatman no. 1 filter paper, containing 2 - 2.5 ml of formatted Dowex, pH 7.0. The technique for formatting Dowex is given in Appendix IV. The Dowex was at neutral pH to allow the negatively charged [¹⁴C]-glutamate to be retained on the column, whilst the neutral [¹⁴C]-glutamine was eluted. The columns were washed by adding 4 x 1 ml of double-distilled water and the effluent collected. 1 ml of the effluent was added to 8 ml of scintillation cocktail (Optiphase HIsafe 3, Pharmacia). [¹⁴C]-glutamine was estimated by scintillation counting (10 minutes per sample, in a Beckman 1701 scintillation counter).

4.2.5. Presentation of data and statistical analysis

Glutamine synthetase activity was expressed as nmol substrate utilised, min⁻¹ g⁻¹ wet weight tissue and as nmol min⁻¹ mg⁻¹ protein (means of the values of protein measured in 1 g of adipose tissue from different depots are listed in Appendix I). Statistical analysis was performed on the means of the data for homologous depots of different depots using Student's t-test and within treatment differences were analysed by ANOVA.

A post-hoc Tukey test was used to test for statistical significance within treatment groups.

4.3. Experiments

4.3.1. The effect of feeding, fasting and exercise on glutamine synthetase activity in extracts of adipose tissue from different sites

Adipose tissue samples used in this study were taken from the same guinea-pigs used in the glutaminase study. For experimental treatments refer to Chapter 2, Section 2.3.2.

4.4. Results

4.4.1. The effect of fasting on glutamine synthetase activity in different adipose sites.

The values of glutamine synthetase activity measured in adipose tissue extracts from the superficial in front of, and behind, fore-limb, and interscapular and from the intermuscular sites, expressed per g wet tissue weight and per mg protein, were not significantly different from each other in the tissue extracts from fasted guinea-pigs (Table 4.1, Figure 4.1). In the intra-abdominal site, omental (adjacent to spleen), glutamine synthetase activity was between 3.5 to 8.5 fold greater than that of any of the other sites measured when expressed per g wet weight, but this difference disappeared when enzyme activity was expressed per mg protein (Table 4.1, Figure 4.1). Glutamine synthetase activity measured per mg protein in adipose tissue extracts from the inguinal site had the highest value of enzyme activity compared to that measured in the superficial sites and omental, mesenteric sites (Table 4.1, Figure 4.1). Extracts of adipose tissue from the mesenteric site after fasting had the lowest mean value of enzyme activity compared with the superficial sites when expressed per g wet weight or per mg protein (Table 4.1, Figure 4.1).

4.4.2. The effect of feeding on glutamine synthetase activity in different adipose sites

Glutamine synthetase activity in extracts of adipose tissue from all of the superficial sites increased significantly directly after the feeding period by approximately 1.5 to 2-fold compared with that of the fasted animals (Table 4.1). Values of enzyme activity remained greater than those measured in the fasted group for up to 30 minutes in the in front of fore-limb and interscapular sites. Glutamine synthetase activity was elevated for up to 240 minutes when expressed per mg protein in adipose tissue extracts from the interscapular site (Table 4.1). In adipose tissue extracts from the remaining superficial sites, mean values of glutamine synthetase activity were greater at 240 minutes after feeding than those of the fasted group, however, the relatively high standard errors precluded statistical significance (Table 4.1).

In tissue extracts from the portally drained mesenteric site, glutamine synthetase activity was not significantly altered by feeding over the time course of 240 minutes (Table 4.1). Although there was a trend for glutamine synthetase activity to be increased after feeding in extracts of adipose tissue from the omentum (adjacent to, and far from, spleen) this change was not statistically significant until 240 minutes following a meal (Table 4.1). In extracts of adipose tissue from the epididymal site, glutamine synthetase activity was increased by approximately two-fold directly after feeding but was not significantly different from the fasted values at the remaining time points (Table 4.1). In extracts of adipose tissue from the perirenal site, glutamine synthetase activity was greater than the fasted values when measured at 60 and 240 minutes after feeding. At the latter time point, the increase in enzyme activity was only significant when expressed per mg protein but not when expressed per g wet weight due to the relatively high standard error (Table 4.1).

In extracts of adipose tissue from the intermuscular sites, cervical and popliteal, glutamine synthetase activity was increased directly after feeding when expressed per mg protein. Glutamine synthetase activity (per mg protein) for these two sites remained elevated for up to 240 minutes although values for 30 minutes in cervical, and 30 and 60

minutes for popliteal, were not significantly different from fasted values due to the relatively high standard errors (Table 4.1).

Table 4.1a Effect of feeding over a time course of 240 minutes on glutamine synthetase activity in extracts of superficial adipose tissue.

Values are mean (\pm SEM) expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet weight and nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein in parentheses. Number of experiments = 5 in fasted group and 4 in each of the fed groups.

The statistical differences between the means of fasted and fed animals are denoted by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ¥ denotes significantly higher ($P < 0.05$ or less) glutamine synthetase activity than most other sites measured at the same time point, • denotes significantly lower ($P < 0.05$ or less) glutamine synthetase activity than most other sites measured at the same time point.

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
In front	119.2 \pm 25.6	268.2 \pm 27.2*	243.9 \pm 36.6*	189.2 \pm 42.4	259.5 \pm 67.6
fore-limb	(16.6 \pm 3.6)	(39.6 \pm 5.9*)	(33.9 \pm 5.1*)	(24.3 \pm 4.9)	(36.1 \pm 10.9)
Behind	159.9 \pm 20.9	382.0 \pm 14.0***	229.7 \pm 61.0	245.0 \pm 53.8	242.8 \pm 79.6
fore-limb	(21.1 \pm 2.8)	(51.7 \pm 4.3***)	(30.2 \pm 5.4)	(31.7 \pm 7.2)	(32.0 \pm 12.1)
Inter- scapular	139.5 \pm 34.4	275.2 \pm 38.4*	301.9 \pm 51.4*	302.5 \pm 63.4	305.0 \pm 92.5
	(20.8 \pm 4.1)	(39.6 \pm 6.1*)	(45.6 \pm 7.6*)	(43.7 \pm 8.3*)	(58.1 \pm 8.1**)
Inguinal	137.6 \pm 21.9	339.5 \pm 97.3*	177.0 \pm 73.8	172.0 \pm 43.3	252.4 \pm 57.0
	(29.5 \pm 3.7¥)	(56.8 \pm 9.8*)	(37.4 \pm 10.9)	(36.6 \pm 6.3)	(53.9 \pm 12.2)

Table 4.1b Effect of feeding over a time course of 240 minutes on glutamine synthetase activity in extracts of intra-abdominal adipose tissue.

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
Omental (adjacent to spleen)	569.3±120 [¥] (16.9±3.6)	731.3±97.7 (23.2±3.4*)	550.2±245.6 (17.4±3.1)	778.4±202.2 (24.6±5.9)	992.5±156.8* (31.4±2.7*)
Omental (far from spleen)	118.9±23.8 (15.0±3.0)	164.1±11.2 (24.3±5.6*)	178.5±20.9 (19.0±5.6)	176.7±34.7 (20.8±3.7)	231.7±64.6 (36.7±4.9**)
Mesenteric	66.2±34.4 (11.5±2.4*)	28.0±5.3 (3.9±0.8*)	47.0±19.3 (9.6±5.7*)	82.9±16.5 (10.2±1.6*)	90.9±22.4 (15.2±2.6*)
Perirenal	128.2±46.8 (20.7±5.7)	225.1±59.9 (34.3±8.7)	309.9±73.0 (39.2±14.9)	304.2±53.9* (46.6±8.1*)	301.1±95.8 (59.5±10.4**)
Epididymal	117.6±33.3 (18.9±5.2)	263.4±70.4* (44.3±11.5*)	168.4±56.6 (27.7±8.9)	151.5±33.6 (23.6±4.4)	142.2±31.3 (28.2±4.8)

Table 4.1c Effect of feeding over a time course of 240 minutes on glutamine synthetase activity in extracts of intermuscular adipose tissue.

	Sedentary fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
Cervical	212.4±70.8 (24.6±6.4)	329.7±46.1 (40.1±5.7*)	369.3±23.7 (45.5±9.1)	332.0±79.3 (48.7±6.4*)	367.9±85.2 (51.9±6.0**)
Popliteal	112.4±75.9 (18.1±8.8)	259.1±67.6 (45.0±10.2*)	227.6±68.9 (29.1±11.7)	209.7±30.3 (35.6±4.6)	254.0±69.4 (47.6±2.8*)

4.4.3 Between-site differences in glutamine synthetase activity with feeding over a time course of 240 minutes

Glutamine synthetase activity measured in adipose tissue from the mesenteric site was significantly lower ($P < 0.001$) than that of all other sites measured directly after feeding (Figure 4.2). Also at this time point, glutamine synthetase activity was significantly lower in adipose tissue extracts from both omental sites (adjacent to and far from spleen) than that all remaining sites except perirenal (Figure 4.2). This pattern of between-site difference in glutamine synthetase activity was maintained at 30 minutes after feeding, however the relatively high standard errors for the means of enzyme activity in perirenal, epididymal and popliteal adipose tissue extracts precluded a statistically significant difference with the omental sites. At 60 and 240 minutes after feeding the mesenteric site continued to have the lowest glutamine synthetase activity compared with all other sites.

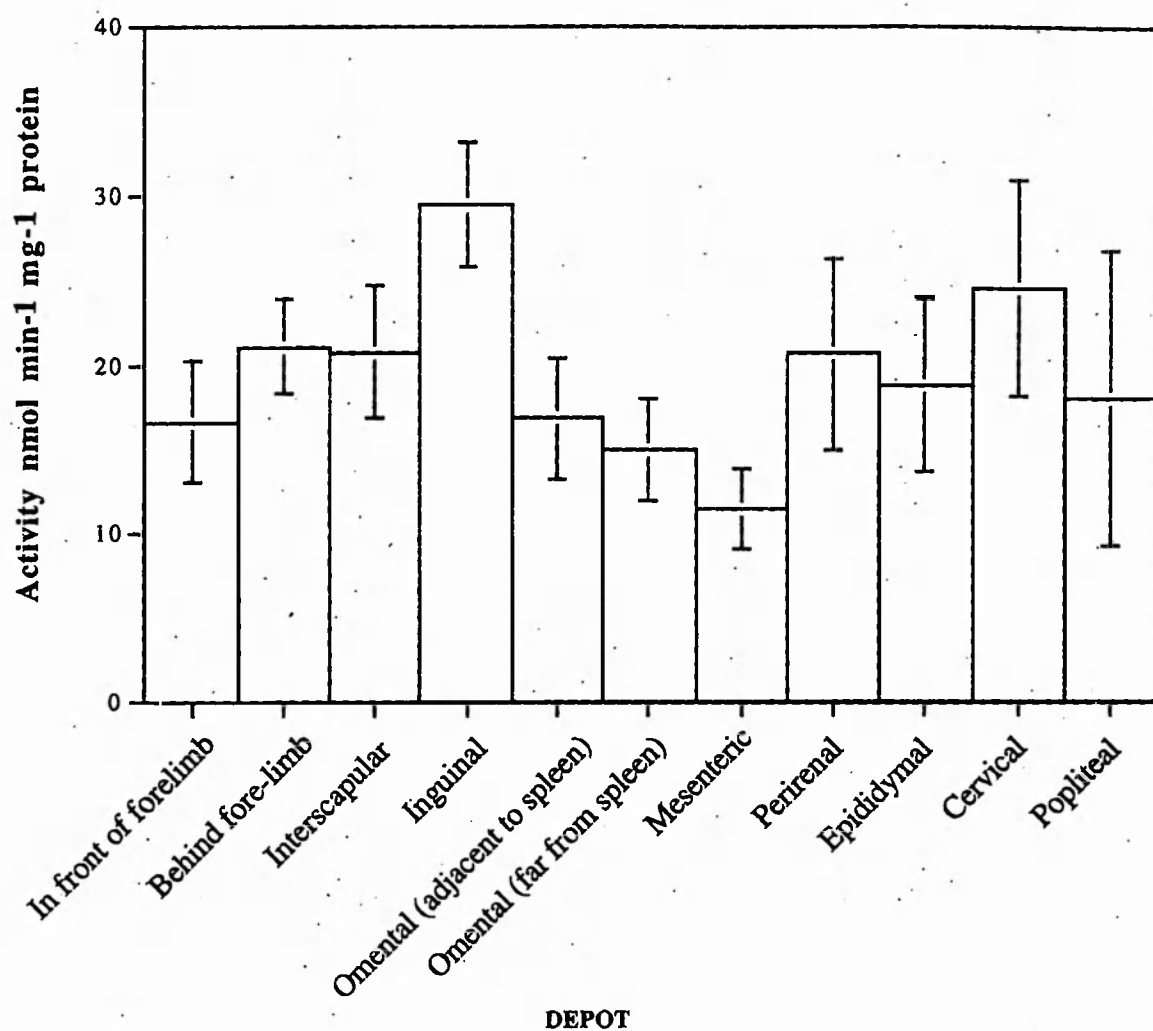


Figure 4.1 Glutamine synthetase activity, in 11 different adipose sites of fasted guinea-pigs, nmol substrate utilised min⁻¹ mg⁻¹ protein.

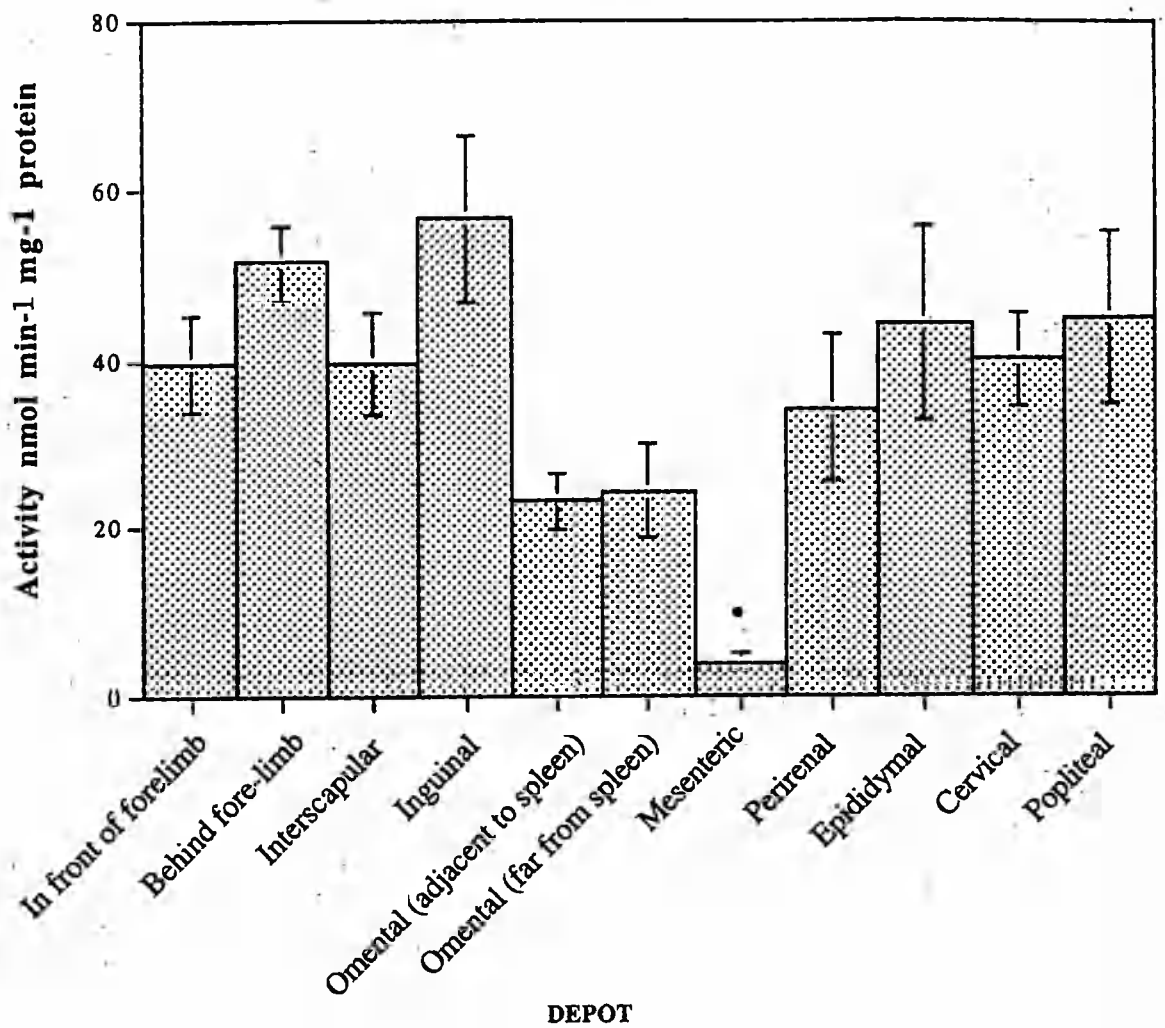


Figure 4.2 Glutamine synthetase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, directly after the feeding period. • denotes significantly lower ($P < 0.05$) than all other sites measured at the same time point.

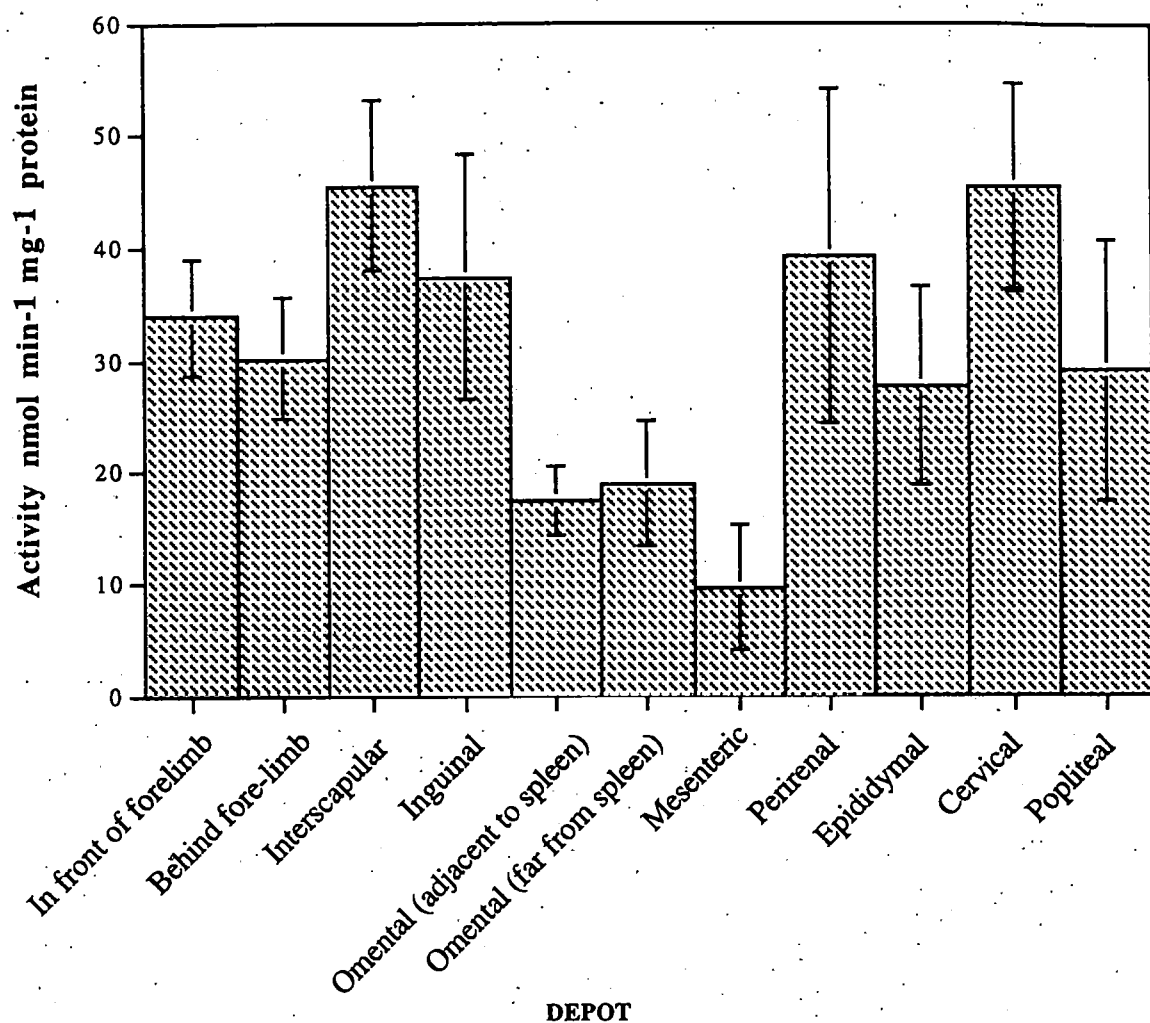


Figure 4.3 Glutamine synthetase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 30 min after the feeding period.

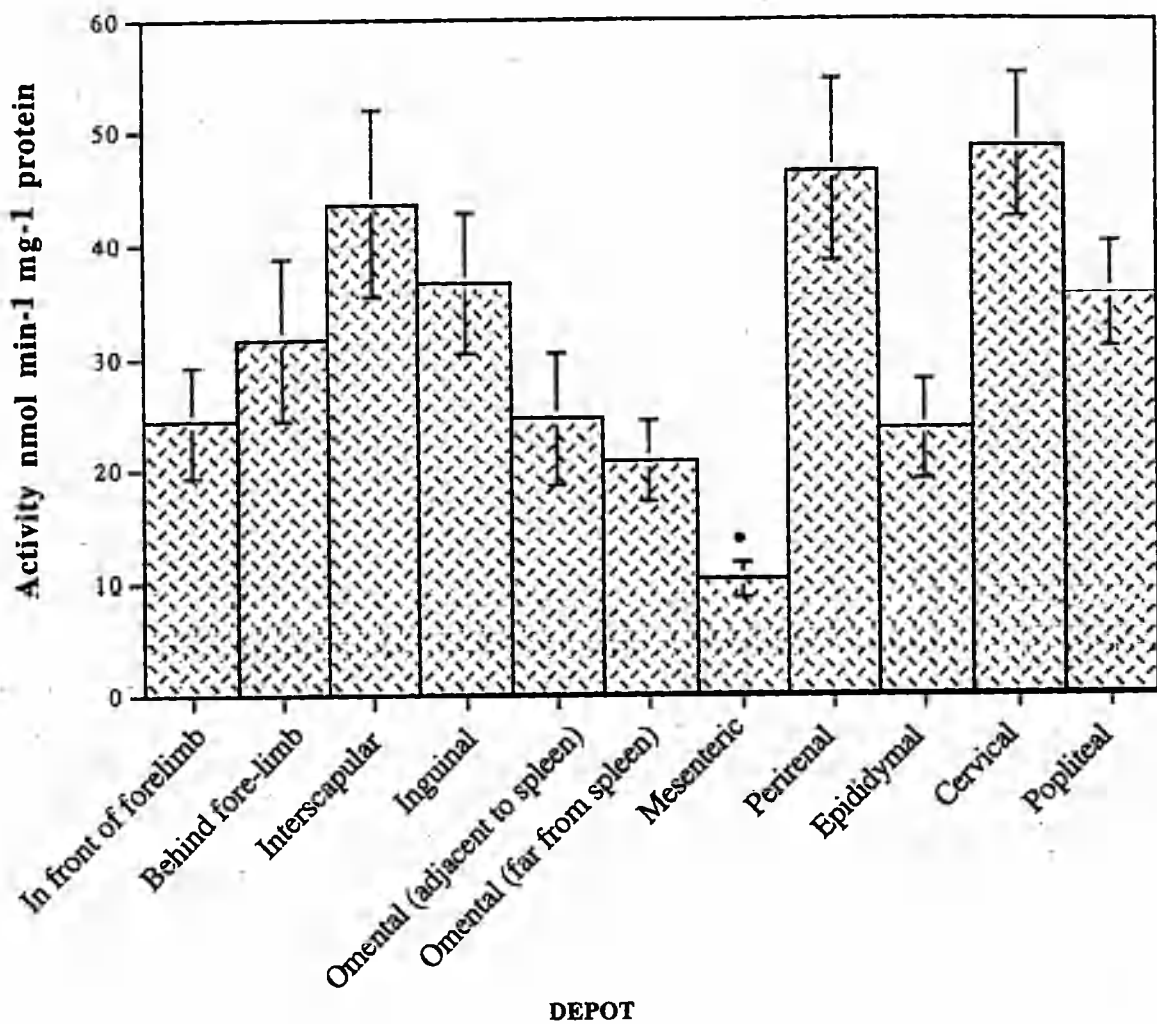


Figure 4.4 Glutamine synthetase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 60 min after the feeding period. • denotes significantly lower ($P < 0.05$) than ~~most~~ other sites measured at the same time point.

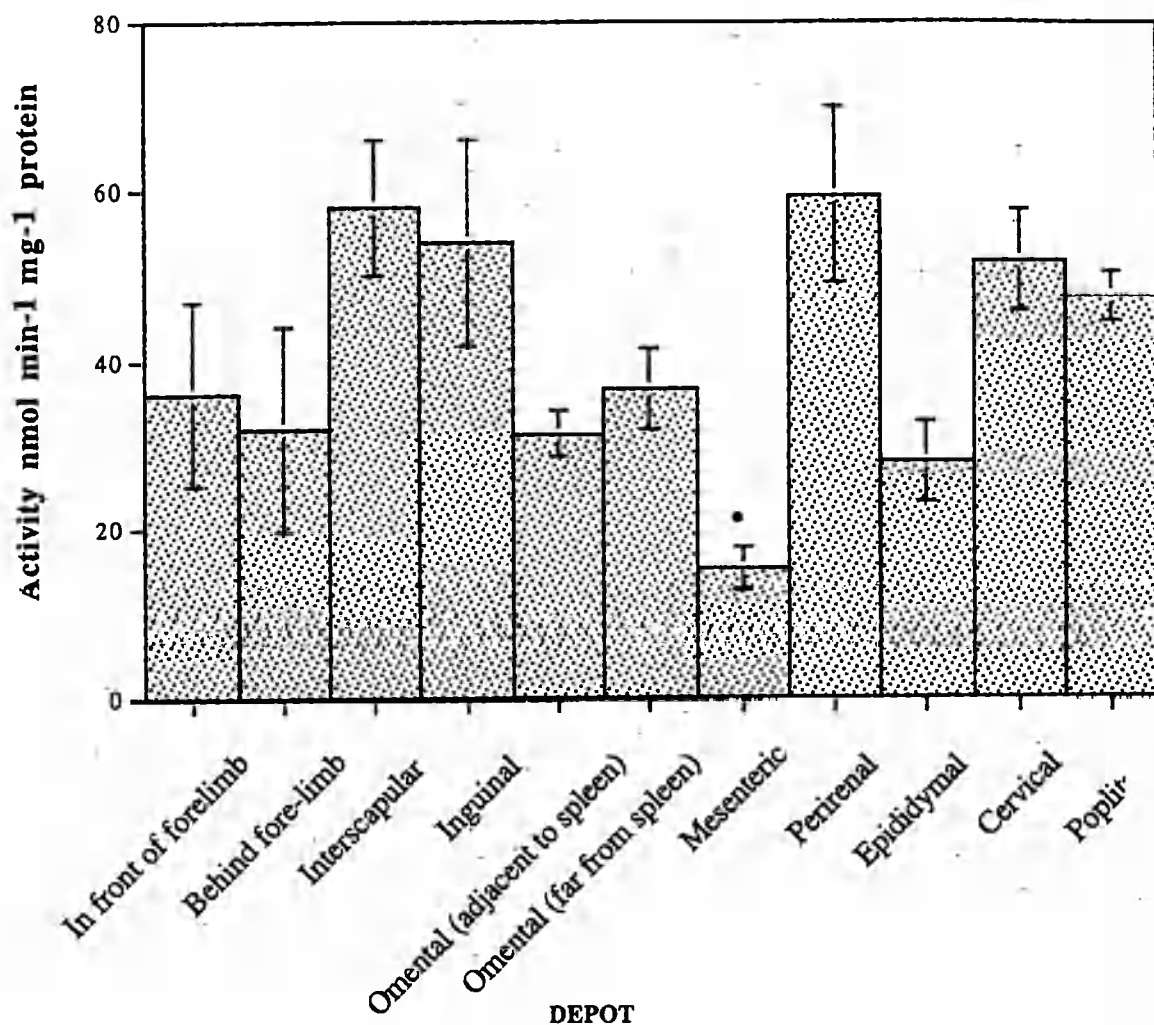


Figure 4.5 Glutamine synthetase activity, in 11 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 240 min after the feeding period. • denotes significantly lower ($P < 0.05$) than ~~not~~ other sites measured at the same time point.

4.4.4 The effect of exercise on glutamine synthetase activity in different adipose sites

The effects of acute exercise on glutamine synthetase activity in adipose tissue extracts were short term and occurred directly after the exercise period. Significant changes in enzyme activity were confined to the intra-abdominal sites (Table 4.2).

In extracts of adipose tissue from the intra-abdominal sites, omental, (adjacent to, and far from, spleen), mesenteric perirenal and epididymal, there was a significant decrease in glutamine synthetase activity directly after exercise, compared with the fasted animals. Values of enzyme activity in all of the intra-abdominal sites were similar to those measured in corresponding tissue extracts from the fasted animals when measured at 30, 60 and 240 minutes after the exercise period (Table 4.5).

Table 4.2a Effect of exercise over a time course of 240 minutes on glutamine synthetase activity in extracts of superficial adipose tissue .

Values are mean (\pm SEM) expressed as nmol substrate utilised $\text{min}^{-1} \text{g}^{-1}$ wet weight and nmol substrate utilised $\text{min}^{-1} \text{mg}^{-1}$ protein in parentheses. Number of separate experiments = 5 in fasted group and 4 in each of the exercised groups.

¥ denotes significantly higher ($P < 0.05$ or less) glutamine synthetase activity than most other sites measured at the same time point, • denotes significantly lower ($P < 0.05$ or less) glutamine synthetase activity than most other sites measured at the same time point.

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
In front fore-limb	119.19 \pm 25.6 (16.6 \pm 3.6)	92.1 \pm 43.1 (12.8 \pm 3.0)	148.0 \pm 26.3 (20.6 \pm 3.7)	136.2 \pm 20.8 (21.8 \pm 3.7)	159.6 \pm 55.5 (22.1 \pm 6.9)
Behind fore-limb	159.9 \pm 20.9 (21.1 \pm 2.8)	104.9 \pm 52.9 (13.8 \pm 3.9)	148.6 \pm 24.1 (19.6 \pm 3.2)	139.3 \pm 20.4 (23.9 \pm 5.2)	167.4 \pm 31.2 (22.1 \pm 4.6)
Inter- scapular	139.5 \pm 34.4 (20.8 \pm 4.1)	89.3 \pm 33.7 (13.3 \pm 5.0)	133.0 \pm 10.4 (19.9 \pm 1.6)	115.0 \pm 18.6 (24.9 \pm 9.1)	148.3 \pm 44.3 (22.2 \pm 3.9)
Inguinal	137.6 \pm 21.9 (29.5 \pm 3.7¥)	176.2 \pm 56.6 (26.1 \pm 8.4¥)	115.5 \pm 14.1 (17.1 \pm 2.1)	148.6 \pm 12.4 (32.6 \pm 4.0)	137.6 \pm 33.1 (29.4 \pm 3.4)

Table 4.2b Effect of exercise over a time course of 240 minutes on glutamine synthetase activity in extracts of intra-abdominal adipose tissue. The statistical differences between the fasted and exercised means are denoted by *P<0.05.

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
Omental (adj. to spleen)	569.3±120 (16.9±3.6)	290.4±24.8* (7.7±0.4*)	732.6±162.5 (21.8±4.8)	271.1±95.6 (13.4±3.4)	445.3±79.6 (13.3±2.7)
Omental (far from spleen)	118.9±23.8 (15.0±3.0)	52.9±26.1* (6.8±3.3*)	137.5±38.2 (17.4±4.8)	125.1±12.2 (21.0±3.2)	125.2±30.1 (15.9±1.7)
Mesenteric	66.2±34.4 (11.5±2.4)	27.2±18.3* (4.1±2.8*)	61.9±16.7 (8.6±2.3*)	99.4±38.7 (8.5±0.8*)	23.6±9.4 (3.3±1.4*)
Perirenal	128.2±46.8 (20.7±5.7)	54.1±20.8* (8.4±2.5*)	103.3±5.4 (16.0±0.8)	134.4±19.9 (27.8±10.1)	127.8±28.9 (20.9±3.2)
Epididymal	117.6±33.3 (18.9±5.2)	31.5±17.8* (6.8±2.9*)	99.2±36.3 (16.0±5.9)	75.8±13.8 (15.2±4.3)	51.3±19.2 (7.9±3.1)

Table 4.2c Effect of exercise over a time course of 240 minutes on glutamine synthetase activity in extracts of intermuscular adipose tissue.

	Sedentary fasted	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
Cervical	212.4±70.8 (24.6±6.4)	117.7±6.7 (13.4±0.8)	142.9±33.3 (16.7±3.9)	157.8±27.4 (24.5±5.4)	129.4±30.0 (14.8±3.3)
Popliteal	112.4±75.9 (18.1±8.8)	67.0±25.6 (13.5±2.7)	90.2±8.8 (15.7±1.5)	127.5±19.6 (24.3±1.03)	155.5±25.9 (22.9±3.7)

4.4.5 Between-site differences in glutamine synthetase activity with exercise over a time course of 240 minutes

Directly after exercise, glutamine synthetase activity was uniformly similar in all adipose sites measured except that measured in inguinal adipose tissue which had the highest enzyme activity (Figure 4.6). At 30, 60 and 240 minutes after exercise, glutamine synthetase activity measured in adipose tissue from the mesenteric site was significantly lower than that any of the remaining sites measured. At the latter time point, after the value for mesenteric adipose tissue, the second lowest glutamine synthetase activity measured was in that of the epididymal site.

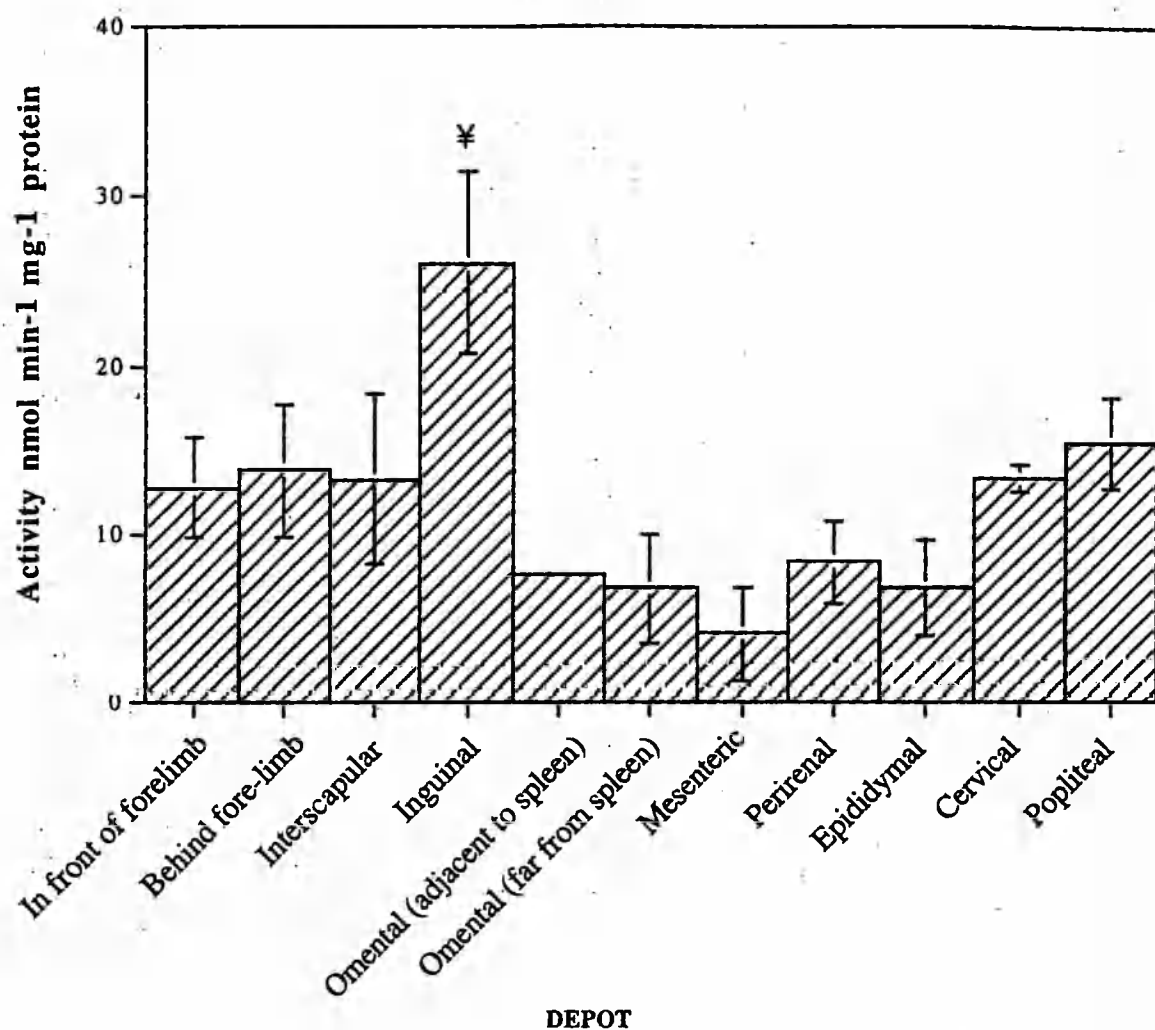


Figure 4.6 Glutamine synthetase activity, in 10 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, directly after exercise. ¥ denotes significantly higher ($P < 0.05$) than all other sites.

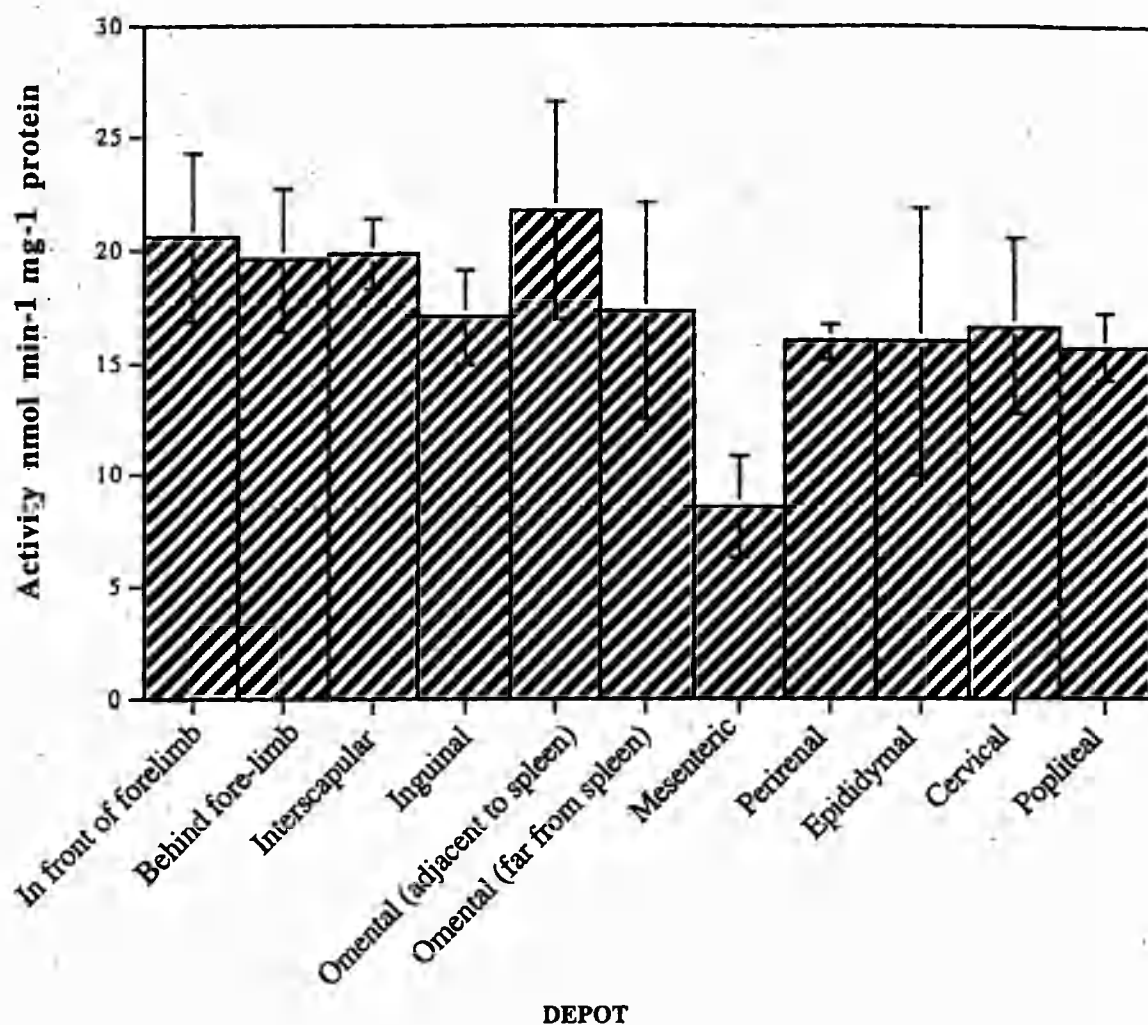


Figure 4.7 Glutamine synthetase activity, in 10 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 30 min after exercise.

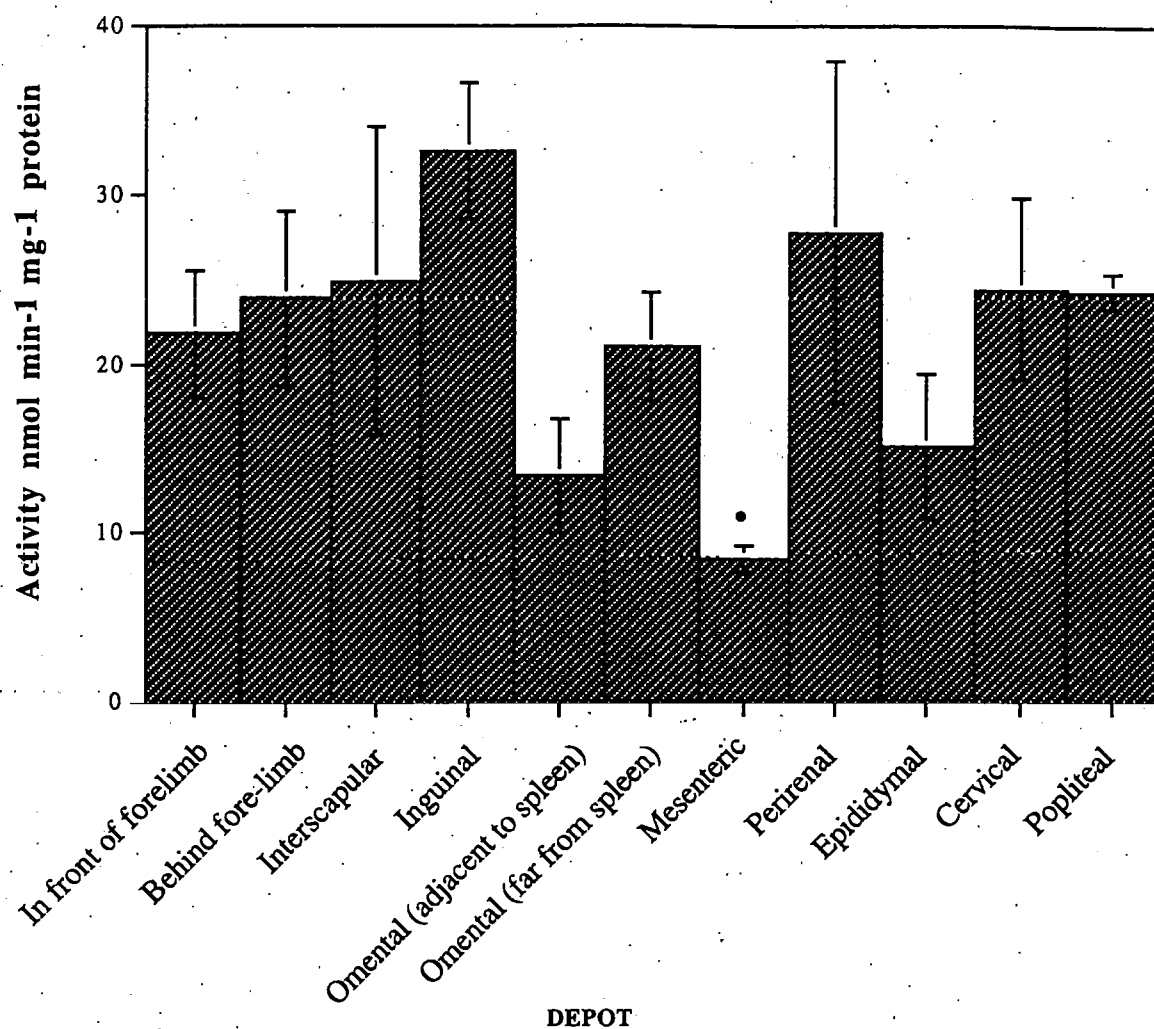


Figure 4.8 Glutamine synthetase activity, in 10 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 60 min after exercise. • denotes significantly lower ($P < 0.05$) than most other sites.

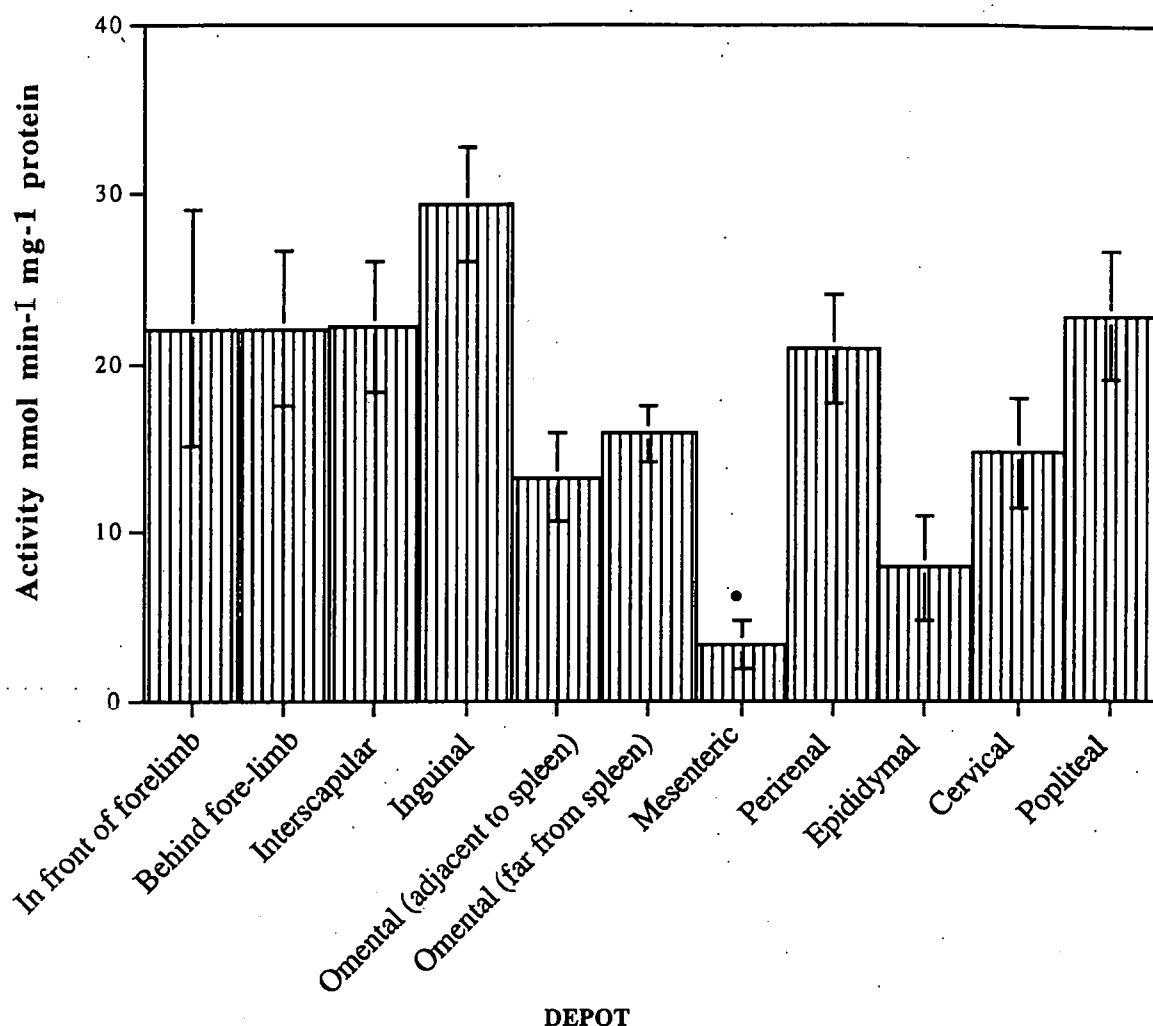


Figure 4.9 Glutamine synthetase activity, in 10 different adipose sites, nmol substrate utilised min⁻¹ mg⁻¹ protein, 240 min after exercise. • denotes significantly lower (P<0.05) than most other sites.

4.5 Discussion.

4.5.1. Glutamine synthetase activity in adipose tissue from different sites after fasting

In the fasted state, glutamine synthetase activity was measurable in extracts of adipose tissue from all sites examined, indicating that the capacity for glutamine synthesis was maintained in this condition. To the author's knowledge, glutamine synthetase has not previously been measured in guinea-pig adipose tissue. However, the means and SE values of enzyme activity reported in this study are within a similar range to those reported by Opara (1993), measured in rat epididymal and perirenal adipose tissue and to those of Watford (1991), measured in rat epididymal adipose tissue. Using the same assay methods, Opara reported values of $404 \pm 44 \text{ nmol min}^{-1} \text{ g wet tissue weight}$ in epididymal adipose tissue and $424 \pm 63 \text{ nmol min}^{-1} \text{ g}$ in perirenal adipose tissue of 6 month old *ad lib*-fed rats weighing 160-250g. Watford reported higher values of $918 \pm 391 \text{ nmol min}^{-1} \text{ g}$ in epididymal adipose tissue of *ad lib* fed rats weighing 250-300 g (age not given).

As with the glutaminase study, values of enzyme activity per g wet tissue, were again greatest in extracts of adipose tissue from the omental (adjacent to spleen site) (see below, Section 4.5.2.). In the fasted state, extracts of adipose tissue from the inguinal site had the highest value of glutamine synthetase activity compared to all other sites when expressed per mg protein (Figure 4.1), as was also the case in the glutaminase study (Chapter 2. Section 2.5.5.). Hence, this large superficial depot has the capacity to contribute significantly to glutamine metabolism in guinea-pigs in the fasted state. The least value of glutamine synthetase activity, per g wet weight and per mg protein, was measured between extracts of adipose tissue from the mesenteric site (Figure 4.1). There were no significant differences in glutamine synthetase activity during fasting in extracts of adipose tissue from the remaining sites. The site-specific differences in the values of glutamine synthetase activity in adipose tissue from fasted guinea-pigs shown in this study are comparable to those of a previous study of glutamine synthetase activity in 4 adipose depots of the fasted rat (Kowalski and Watford 1994b). This study reported a higher value

of glutamine synthetase activity in inguinal adipose tissue compared to mesenteric but little difference in the values of enzyme activity between epididymal and retroperitoneal adipose tissue.

4.5.2. Glutamine synthetase activity in extracts of adipose tissue from the greater omentum, adjacent to the spleen

As with the measurements for glutaminase activity in tissue extracts from this area of the omentum (discussed in Chapter 2, Section 2.5.3), glutamine synthetase activity per g wet weight was significantly higher than those measured in all other sites in both the fasted and fed animals (Table 4.1b). Furthermore, the mean values of glutamine synthetase, per g wet weight, measured in omental tissue extracts (adjacent to spleen) from the fasted animals were significantly greater than those reported for rat skeletal muscle; approximately $550 \text{ nmol min}^{-1} \text{ g}^{-1}$ in omental (adjacent to spleen) compared with $280 \text{ nmol min}^{-1} \text{ g}^{-1}$ in the muscle (data for glutamine synthetase activity in muscle from Opara 1993). Again, the relatively high values of enzyme activity may be attributable to lymphoid tissue present within the adipose tissue as cells of the immune system have also been shown to possess glutamine synthetase activity (Opara 1993). Additionally, mean glutamine synthetase activity measured in pure adipose tissue extracts from the omental (far from spleen) site was much lower than that measured in tissue extracts from the adjacent to spleen site indicating that the high values of enzyme activity found in the latter was more likely to be due to cell types other than adipocytes. Nonetheless, this study shows that this region of the omentum has a significant capacity to synthesise glutamine through the action of glutamine synthetase.

4.5.3. Site-specific effects of fasting, feeding and exercise on the capacity of adipose tissue to synthesise glutamine through the action of glutamine synthetase

The increase in glutamine synthetase activity observed directly after the feeding period in extracts of adipose tissue from epididymal and the superficial and intermuscular

sites but not the remaining intra-abdominal sites shows that there was a differential response by adipose tissue to the effects of a meal. The significant increases in glutamine synthetase activity in extracts of adipose tissue from all of the superficial sites directly after feeding was maintained in the in front of fore-limb and interscapular sites for up to 60 and 240 minutes respectively. The changes in enzyme activity immediately after feeding, and for up to 240 minutes, following the feeding period suggests that in these sites, there is an increase in the capacity to synthesise glutamine under post-prandial conditions.

It is noteworthy that similar treatment had the opposite effect on glutaminase activity in the superficial sites (Chapter 2) (with the exception of the behind fore-limb site). The values of glutaminase activity were significantly reduced directly after feeding to almost non-detectable levels before increasing to above that of the fasted levels at 30 minutes after a meal. The contrasting alterations in the activities of glutamine synthetase and glutaminase directly after the feeding period in these sites would favour glutamine synthesis and possibly its secretion.

There were also increases in glutamine synthetase activity in adipose tissue from the perirenal site 60 minutes after feeding and in the omental sites (adjacent to, and far from, the spleen) 240 minutes after a meal, indicating that these sites were much slower to respond to the effects of a meal than those from the superficial and intermuscular sites.

Adipose tissue from the portally drained mesenteric site showed no significant alterations in glutamine synthetase activity after a meal over the time course studied and enzyme activity was consistently lower than that measured in all other sites. Conversely, glutaminase activity was increased directly after feeding in adipose tissue extracts from the omental (far from spleen) and mesenteric sites suggesting that glutamine utilisation in these portally drained sites is increased but synthesis is not affected until 240 minutes after a meal then only in the omental sites. Stimulation of adipose tissue glutamine synthetase activity *in vivo* could involve alterations of mRNA and protein synthesis or post-translational modifications of the enzyme. In 3T3-L1 preadipocytes, the glutamine synthetase gene contains potential hormone-response elements including glucocorticoid (GRE) and cAMP response elements (CRE) (Bhandari *et al.* 1988). Glutamine synthetase

mRNA has been shown to have a relatively short half-life of 95 to 110 minutes (Saini *et al.* 1990) which could provide a burst of protein synthesis in response to internal or external stimuli. The glucocorticoid analogue, dexamethasone causes a 10-fold increase in glutamine synthetase mRNA abundance and a 2-fold increase in glutamine synthetase gene transcription in 3T3-L1 preadipocytes (Miller and Burns 1985). However, since serum cortisol levels in the fed guinea-pig were reduced compared with that of the fasted animals (Chapter 3, Section 3.4.5.) it is unlikely that this hormone promotes mRNA abundance and gene transcription with feeding.

Insulin levels in the circulation are likely to increase with the onset of feeding so action of this hormone on glutamine synthetase mRNA abundance and stability should be considered. Treatment of fully differentiated 3T3-L1 adipocytes with supra-physiological concentrations of insulin results in reduced glutamine synthetase biosynthesis compared to that of cells incubated without insulin or with dexamethasone (Miller *et al.* 1983). However, to the author's knowledge, there are no data on the effect of insulin on glutamine synthetase mRNA in mature adipocytes.

4.5.4. Glutamine synthetase activity in adipose tissue extracts after exercise over a time course of 240 minutes

A significant effect of brief but acute exercise on glutamine synthetase activity in adipose tissue was confined to the intra-abdominal sites omental, mesenteric, perirenal and epididymal, where reductions in enzyme activity compared with the fasted values occurred directly after the exercise period. Values of enzyme activity in these sites returned to fasted levels by 30 minutes, and in adipose tissue from the mesenteric site, glutamine synthetase activity remained low compared to the remaining sites measured at 30, 60 and 240 minutes. Glutamine synthetase activity in adipose tissue extracts from the superficial and intermuscular sites remained unchanged after exercise compared with that of the fasted animals, showing a differential response by these sites to factors which may modulate this enzyme in intra-abdominal adipose tissue.

The differential alterations in the intra-abdominal sites of glutamine synthetase activity directly after exercise may, in part, be explained an enhanced β -adrenergic mediated response to catecholamines. In humans, it has been shown that omental adipocytes have significantly more β -adrenergic receptors than subcutaneous abdominal adipocytes (Hellmer *et al.* 1992) which may partly explain why catecholamines have a greater effect on lipolysis in omental adipose tissue compared to subcutaneous adipose tissue (reviewed by Smith 1985). In the guinea-pig, mesenteric and omental adipocytes have a greater lipolytic response to noradrenaline than superficial inguinal and behind forearm sites (Pond and Mattacks 1991). As with humans, differences in the β -adrenergic response to catecholamines in guinea-pigs may partly explain the site-specific differences in lipolysis. The reduction in glutamine synthetase activity in adipose tissue may therefore have been effected by an elevation in the level of intracellular cAMP which has been shown to cause significant reductions in mRNA stability, gene transcription and catalytic activity of this enzyme in 3T3-L1 adipocytes (Miller *et al.* 1978). Hormones which serve to increase intracellular levels of cAMP include the catecholamines, noradrenaline and adrenaline. Although no measurements circulating hormone concentrations were made before and after exercise in this study, it is likely that the levels of these hormones would be elevated due to the stressful nature of the exercise regime (discussed in Chapter 2, Section 2.5.7).

Other factors which may have served to reduce glutamine synthetase activity in the intra-abdominal sites could include a reduction in the delivery of substrates necessary for glutamine synthesis to proceed. In the study described in Chapter 2, glutaminase activity in extracts of adipose tissue from omental (far from spleen), epididymal and perirenal sites was virtually abolished directly following exercise. Therefore, at this time point intracellular availability of glutamate for transamidation may be significantly reduced. In addition, hepatic blood flow in humans is decreased with exercise, which serves to divert blood to skeletal muscle, and may also restrict the delivery of substrates for glutamine synthesis in intra-abdominal adipose tissue.

An enhanced lipolytic response to catecholamines in omental and mesenteric adipose tissue, coupled to reductions in the capacity for these sites to utilise and synthesise glutamine, would therefore place the emphasis on the release of lipids rather than amino acids from these sites.

4.5.5. Estimation of the *in vivo* activity of glutamine synthetase in adipose tissue after fasting feeding and exercise

Using the Michaelis-Menten equation, it is possible to calculate the approximate *in vivo* activity of glutamine synthetase in adipose tissue from different sites thus giving an estimation of the overall capacity for adipose tissue to synthesise glutamine.

Michaelis Menten equation:

$$v = \frac{V_{\max} [S]}{[S] + K_m}$$

where v = '*in vivo*' enzyme activity

V_{\max} = maximal enzyme activity

$[S]$ = substrate concentration

Miller (1975) determined the K_m value for glutamine synthetase in rat adipose tissue to be 2 mM. Endogenous glutamate in guinea-pig adipose tissue extracts was measured in the control samples of the glutaminase study described in Chapter 2 and was found to range from 0.1 to 0.16 mM with the exception of omental adjacent to spleen site where the glutamate concentration was estimated to be 0.5 mM.

Using the Michaelis Menten equation, the '*in vivo*' activity of glutamine synthetase was calculated per g of adipose tissue for each adipose site after fasting and feeding (Table 4.3) and exercise (Table 4.4). The total activity for each site was then calculated by multiplying the '*in vivo*' activity per g by the estimated mass of each depot (Table 4.3). From these calculations, adipose sites which had the capacity to contribute most in the fasted state to the supply of glutamine '*in vivo*' were, the relatively large interscapular, inguinal and perirenal depots and the much smaller omental (adjacent to spleen) site. The total estimated '*in vivo*' activity, assuming that guinea-pig adipose tissue

mass represents 15% of total body weight, was $1.57 \mu\text{mol}$, or $10.5 \text{ nmol min}^{-1} \text{ g}^{-1}$ wet tissue weight in the fasted state. After feeding, the estimated '*in vivo*' activity of glutamine synthetase increased in all sites over the time course of 240 min, except mesenteric where values were reduced directly following, and at 30 minutes after feeding compared to the fasted values. The estimated '*in vivo*' activity was $2.6 \mu\text{mol min}^{-1}$ for total adipose tissue mass in the fed animals or $17.5 \text{ nmol min}^{-1} \text{ g}^{-1}$ wet weight. At 30 minutes after feeding '*in vivo*' activity decreased slightly to $2.4 \mu\text{mol min}^{-1}$ for total adipose tissue mass and $15.8 \text{ nmol min}^{-1} \text{ g}^{-1}$. At 60 minutes after feeding, glutamine synthetase activity '*in vivo*' increased to $3.6 \mu\text{mol min}^{-1}$ for total adipose tissue mass and $24.2 \text{ nmol min}^{-1} \text{ g}^{-1}$, and after 240 minutes '*in vivo*' activity was $2.9 \mu\text{mol min}^{-1}$ for total adipose tissue mass and $19.7 \text{ nmol min}^{-1} \text{ g}^{-1}$. To the author's knowledge there are no comparable data for glutamine synthesis and release from guinea-pig adipose tissue. However, these estimated values of '*in vivo*' glutamine synthetase activity are comparable to the '*in vivo*' release of glutamine from inguinal adipose tissue of *ad libitum* fed rats (Kowalski and Watford 1994a) which was estimated to be $24 \text{ nmol min}^{-1} \text{ g}^{-1}$ wet tissue weight. The estimated values of '*in vivo*' glutamine synthetase activity in guinea-pig epididymal adipose tissue of $8.2 \text{ nmol min}^{-1} \text{ g}^{-1}$ (Table 4.3) also compare closely with the '*in vitro*' glutamine release of $7.2 \text{ nmol min}^{-1} \text{ g}^{-1}$ from rat epididymal adipose tissue reported by Ardawi (1988). Therefore, the '*in vivo*' values of glutamine synthetase calculated from this study are comparable to both '*in vivo*' and '*in vitro*' reported values of glutamine release from adipose tissue, indicating a direct relationship between the enzymatic capacity for synthesis and release of glutamine from this tissue.

The increase in the capacity for adipose tissue to synthesize glutamine through enhanced '*in vivo*' activity of glutamine synthetase after feeding (Table 4.3) is in agreement with the study by Tischler & Goldberg (1980). These authors found that adipose tissue from the fed rat releases significant quantities of glutamine '*in vitro*' and when incubated with the branch chain amino acids (leucine, isoleucine and valine) and insulin a greater quantity was released compared with that measured in adipose tissue from fasted rats under the same conditions. From this study, Tischler & Goldberg concluded that adipose

tissue has the capacity to contribute significantly to circulating glutamine in the fed state and that this capacity falls with fasting.

Exercise resulted in an overall decrease in the estimated '*in vivo*' activity of glutamine synthetase to $964 \text{ nmol min}^{-1}$ for the total adipose tissue mass and $6.4 \text{ nmol min}^{-1} \text{ g}^{-1}$ wet weight (Table 4.4) compared with that of the fasted animals (1570 and $11.5 \text{ min}^{-1} \text{ g}^{-1}$ respectively). However, by 30 minutes the estimated '*in vivo*' value of glutamine synthetase activity in adipose tissue had returned to that of the fasted animals. The changes in enzyme activity directly after exercise demonstrate a transient downward shift in the capacity of adipose tissue to synthesize glutamine.

Table 4.3 *In vivo* glutamine synthetase activities in different adipose sites with fasting and feeding, values calculated from Tables 4.1a, 4.1b and 4.1c. Values represent *in vivo* glutamine synthetase activity, nmol min⁻¹ g⁻¹ wet weight, and in parentheses, nmol min⁻¹ total adipose mass of each site.

	Fasted	Fed	Fed + 30 min	Fed + 60 min	Fed + 240 min
In front fore-limb	8.3 (75)	18.7 (168)	17.0 (153)	13.2 (119)	18.1 (163)
Behind fore-limb	11.1 (151)	26.6 (360)	16.0 (216)	17.1 (230.8)	16.9 (229)
Inter- scapular	9.7 (219)	19.2 (432)	21.1 (474)	21.1 (475)	21.2 (479)
Inguinal	9.6 (230)	23.7 (569)	12.3 (296)	12.0 (288)	17.6 (422)
Omental (adj.spleen)	132.4 (265)	170.7 (340)	127.9 (256)	181.0 (362)	230.8 (462)
Omental (far spleen)	8.3 (33)	14.4 (46)	12.4 (50)	12.3 (49)	16.1 (65)
Mesenteric	4.6 (104)	1.9 (44)	3.3 (74)	5.8 (130)	6.3 (143)
Perirenal	8.9 (282)	15.7 (495)	21.6 (681)	21.2 (669)	21.0 (662)
Epididymal	8.2 (86)	18.3 (193)	11.7 (123)	10.5 (111)	9.9 (104)
Cervical	14.8 (89)	23.0 (138)	25.7 (155)	22.3 (134)	25.6 (154)
Popliteal	7.8 (35)	18.1 (82)	15.9 (71)	14.5 (66)	17.7 (80)
Total g⁻¹ (adipose mass)	11.5 (1570)	17.5 (2628)	15.8 (2371)	24.21 (3632)	19.7 (2962)

Table 4.4. *In vivo* glutamine synthetase activities in different adipose sites with exercise, values calculated from Tables 4.2a, 4.2b and 4.2c. Values represent *in vivo* glutamine synthetase activity, nmol min⁻¹ g wet weight, and in parentheses, nmol min⁻¹ total adipose mass of each site.

	Exercised	Exercised + 30 min	Exercised + 60 min	Exercised + 240 min
In front forelimb	6.4 (58)	10.3 (93)	9.5 (86)	11.1 (100)
Behind forelimb	7.31 (99)	10.4 (140)	9.7 (131)	11.7 (158)
Inter- scapular	6.23 (140)	9.3 (209)	8.0 (181)	10.3 (233)
Inguinal	12.3 (295)	8.1 (193.4)	10.3 (249)	9.6 (230)
Omental (adj.spleen)	67.5 (135)	170.4 (341)	63.0 (126)	103.6 (207)
Omental (far spleen)	3.7 (15)	9.6 (38.)	8.7 (35)	8.7 (35)
Mesenteric	1.9 (43)	4.3 (97)	6.9 (156)	1.6 (37)
Perirenal	2.2 (69)	6.9 (218)	5.3 (167)	3.6 (113)
Epididymal	3.8 (40)	7.2 (76)	9.4 (98)	8.9 (94)
Cervical	8.2 (49)	10.0 (60)	11.0 (66)	9.0 (54)
Popliteal	4.7 (21)	6.3 (28)	8.9 (40)	10.8 (49)
Total g⁻¹ (Adipose mass)	6.4 (964)	10.0 (1493)	8.9 (1334)	8.7 (1310)

4.5.6. Glutamine synthetase activity in adipose tissue compared with that in skeletal muscle

To the author's knowledge, there are no reports on glutamine synthesis and release by guinea-pig muscle. However, in a study by Ardawi & Jamal (1990), rat skeletal muscle *in vitro* was shown to release 12.9 nmol glutamine min⁻¹ g wet tissue weight⁻¹. The estimated '*in vivo*' glutamine synthetase activity in guinea-pig adipose tissue from fasted and fed animals (Table 4.3) in the present study indicate that adipose tissue per g wet weight has the capacity to release as much as that of rat muscle per g wet weight, assuming that values of synthesis and release are similar.

A comparison of the '*in vivo*' glutamine synthetase activity of adipose tissue with that estimated in muscle of the guinea-pig shows that, in terms of the whole body, muscle, because of its mass, has the capacity to synthesise more glutamine than adipose tissue. However, the data from the present study indicate that adipose tissue may contribute to approximately 30% of the whole-body glutamine release compared to that of muscle in the fed state. The estimates of glutamine release based on '*in vivo*' enzyme activity in adipose tissue are in agreement with that of Frayn *et al.* (1991) who calculated that human adipose tissue may contribute up to one third as much as muscle to whole body circulating glutamine supply.

4.5.7. Conclusion

The activities of glutamine synthetase measured after feeding, fasting and exercise demonstrate that adipose tissue has the capacity to contribute significantly to whole body glutamine metabolism. This finding is in agreement with previous findings (Tischler and Goldberg 1980, Frayn *et al.* 1991 and Kowalski and Watford 1994a). Furthermore, analysis of glutamine synthetase activity in all the major adipose depots in this study provides additional information about the capacity of adipose tissue to synthesize glutamine in that the responses to fasting and feeding are not uniform throughout the different depots. This factor demonstrates the need to measure enzyme activity in all of the

major adipose depots so that site-specific differences in the responses to differing physiological conditions over a time course can be accounted for.

Chapter 5

**The effects of insulin,
dexamethasone and isoprenaline on
glutamine release from isolated
adipocytes prepared from five
different adipose depots**

5.1. Introduction

Previous *in vitro* and *in vivo* studies have shown that adipose tissue from humans and rats has the capacity to synthesise and release significant quantities of glutamine into the circulation (Christophe *et al.* 1966, Tischler and Goldberg 1980, Frayn *et al.* 1991 and Kowalski and Watford 1994a). Tischler and Goldberg (1980) measured the release of glutamine in the presence of BCAA by whole epididymal fat pads taken from fed and fasted rats. This study demonstrated that fat pads taken from both the fed and fasted animals released glutamine and that the rate of release was increased when leucine was added to the incubation medium. Furthermore, the relative amounts of glutamine released were greater in adipose tissue taken from the fed compared with the fasted animals. This study also showed that addition of insulin to the incubation medium resulted in an increase in both the breakdown of leucine and the release of glutamine. It was concluded therefore that adipose tissue has the capacity to contribute significantly to circulating glutamine levels in the fed state and to a lesser extent during fasting. In addition, the degradation of leucine is coupled to the synthesis and release of glutamine from adipose tissue. Further *in vivo* studies measured the exchange of alanine, glutamate and glutamine across human abdominal subcutaneous and rat inguinal adipose tissue by microdialysis techniques (Frayn *et al.* 1991 and Kowalski and Watford 1994a). Both these studies demonstrated a net production of glutamine and alanine with an uptake of glutamate by subcutaneous adipose tissue. In agreement with Tischler and Goldberg (1980), it was concluded that adipose tissue has a significant capacity to contribute to circulating glutamine, particularly in humans, as adipose tissue can constitute a large percentage of the total body weight. In all of the above studies, extrapolations of the adipose tissues' total contribution to whole body glutamine supply were made using data from only one adipose depot.

In the study of glutamine synthetase activity described in Chapter 4, there were some site-specific differences in the activity of this enzyme and in its response to feeding, fasting and exercise. These findings indicate that adipocytes from different anatomical locations have a differential capacity to synthesise glutamine which may ultimately affect

both the local supply to adjacent tissues and the total contribution of this tissue to circulating glutamine levels.

5.1.1. Hypothesis

The basal rate of release of glutamine from isolated adipocytes differs between depots and is (in part) regulated by the hormones released during feeding, fasting and exercise. The rate of release may also be enhanced by branch chain amino acids, specifically, leucine. The effect of hormones on both the catalytic activity of glutamine synthetase and the subsequent release of glutamine from different adipose sites plays a role in maintaining whole-body glutamine homeostasis with altering physiological status.

5.1.2. Aims of study.

The aims of this study were to measure how the hormones (or analogues of hormones) detailed in Chapter 3, insulin, dexamethasone and isoprenaline, which would be expected to be increased with feeding, fasting, and exercise respectively, altered glutamine release from isolated adipocytes prepared from the five different adipose sites of fasted guinea-pigs. The results from the glutamine synthetase study detailed in Chapter 4 were then compared to determine whether the site-specific alterations in catalytic activity observed with different physiological conditions correlate with the effects of insulin, dexamethasone and isoprenaline on glutamine release from isolated adipocytes prepared from different adipose sites.

Furthermore, as glutamine release from epididymal adipose tissue *in vitro* has been shown to be increased in the presence of the branch chain amino acid leucine (Tischler and Goldberg 1980) its effect on release was measured in adipocytes isolated from different adipose sites to determine whether the overall site-specific action of this amino acid was similar.

5.2. Materials and methods

5.2.1. Animals and dissection procedure.

Adipose tissue was obtained from 6 month old male Bolivian guinea-pigs (body mass 795 ± 16 g) born and raised at the Open University. The animals were fasted overnight for 18 hours then sacrificed between 0830 and 0930 with an intra-peritoneal injection of 3% pentobarbital dissolved in phosphate buffered saline.

Dissection procedures and the adipose sites used for this study were the same as those described in Chapter 3. (Section 3.2.1).

5.2.2. Materials.

The chemicals and enzymes are detailed in Appendix II.

5.2.3. Preparation of adipocytes

Adipocytes were prepared from inguinal, mesenteric, omental, popliteal and epididymal adipose sites. The method used for isolation of the adipocytes is described in Chapter 3 (Section 3.2.6).

5.2.4. Preparation of incubation media.

The incubation media consisted of KRB supplemented with BSA (0.2%) and glucose (5.6 mM), pH 7.4, warmed to 37 °C and gassed for 30 minutes with O₂, 95%/CO₂, 5% prior to the addition of BSA and glucose. The buffer was then divided into preincubation and incubation media.

5.2.5. A study of glutamine release from isolated adipocytes from different adipose sites over a time course of 60 minutes

Adipocytes isolated from the above mentioned sites were washed as described in Chapter 3 (Section 3.2.6.). Aliquots (300 μ l) were added to 1.5 ml Eppendorf tubes containing 700 μ l of preincubation buffer. The cells were preincubated at 37 °C for 30 minutes under a constant stream of O₂ 95%/CO₂ 5%. Following preincubation the buffer was aspirated off using a 2 ml plastic syringe. Incubation media (700 μ l) was added to the cells which were incubated for 5, 15, 30 and 60 minutes at 37 °C under a stream of O₂ 95%/CO₂, 5%. After the appropriate incubation period the reaction was terminated by the addition of 100 μ l of ice-cold ouabain (10 μ M). The samples were then placed immediately on ice. The adipocytes were separated from the incubation buffer through di isononyl phthalate as described in Chapter 3 (Section 3.2.7.). The incubation media were retained and stored at -20 °C for subsequent analysis of glutamine concentration. Cell viability was checked at the end of the 60 minute incubation period using the Trypan blue exclusion method.

5.2.6. The effect of leucine on glutamine release from isolated adipocytes over a time course of 60 minutes

Isolated adipocytes were prepared and preincubated as described in Section 5.2.4. Leucine (1 mM) was added to the incubation media and adipocytes were incubated for 5, 15, 30 and 60 minutes. The reaction was terminated and the media were retained for determination of glutamine release as described above.

5.2.7. Preparation of insulin, dexamethasone and isoprenaline

Preparations of stock solutions of insulin, dexamethasone, and isoprenaline are detailed in Appendix II. The final concentrations of hormones (or their analogues) used in the incubation media were: insulin, 50 mU ml⁻¹, dexamethasone, 2.5 μ M, isoprenaline, 1.0 μ M.

5.2.8. Measurement of glutamine release into the incubation media.

Release of glutamine from isolated adipocytes was measured by spectrophotometer (Pye-Unicam 8625) using a modified method of Windmueller and Spaeth (1974).

Principle: Use is made of the ability of glutamate dehydrogenase to aminate α -ketoglutarate reductively. Amido-hydrolysis provides the requisite ammonia for this condensation. In the process NADH is oxidised with the attendant fall in extinction at 340 nm. Asparaginase is used to catalyse these amidohydrolyses.

Composition of the assay medium:

Chemical	Final concentration in 1 ml
KH ₂ PO ₄	45 mM
glycerol	50%
NADH	17.34 mM
BSA (Fraction V)	10 %
Glutamate dehydrogenase	20 units
*Asparaginase	20 units
2-oxoglutarate	3.6 mM

(*Asparaginase was dialysed over 24-36 hours in 1500 ml phosphate buffer (80 mM, pH 6.6) at 4°C. The buffer was changed every 12 hours. This process removed any ammonia present in the enzyme preparation).

Standard solutions of glutamine and a water blank were included in the assay.

Glutamine assay method:

In a 1 ml cuvette, 960 μ l of assay medium (not including asparaginase) was mixed with 100 μ l of the sample and incubated at room temperature for 30 minutes. Absorbances for each sample, standard and blank were measured at 340 nm. Asparaginase (20 units) was then added to each sample; mixed and incubated at room temperature for 60 minutes at which time a second absorbance measurement was taken at 340 nm. Glutamine concentration was determined by the stepwise decrease in absorbance at 340 nm.

5.2. 9. Presentation of data and statistical analysis

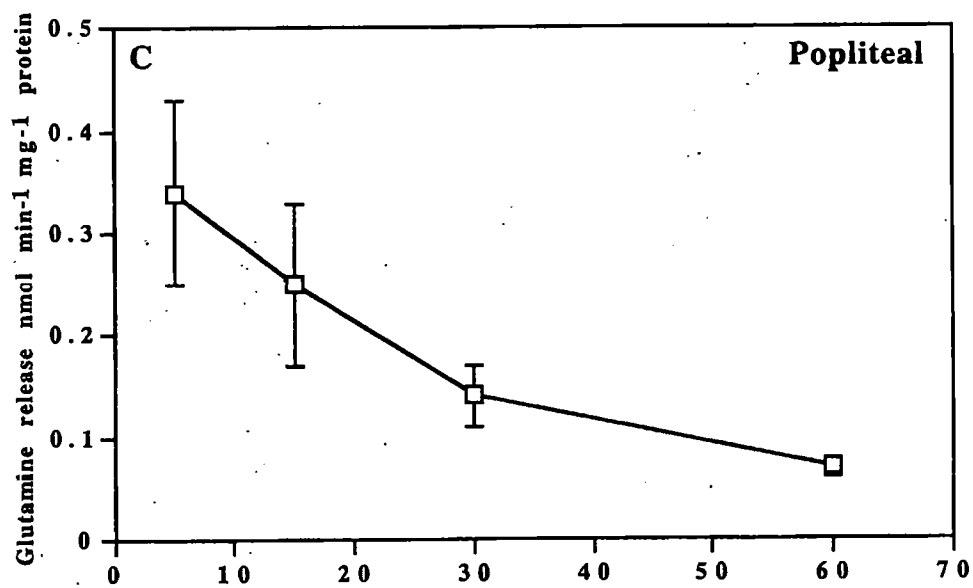
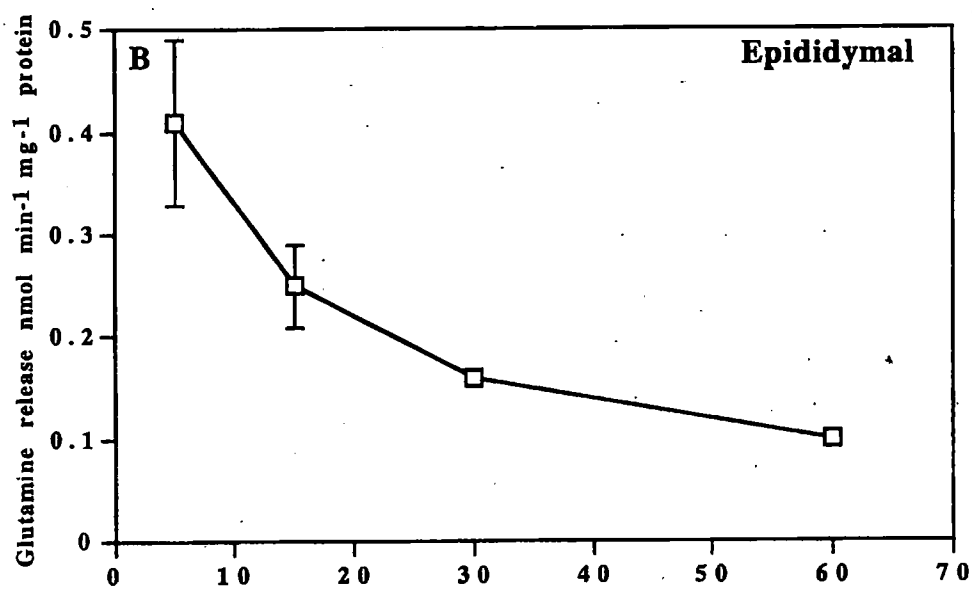
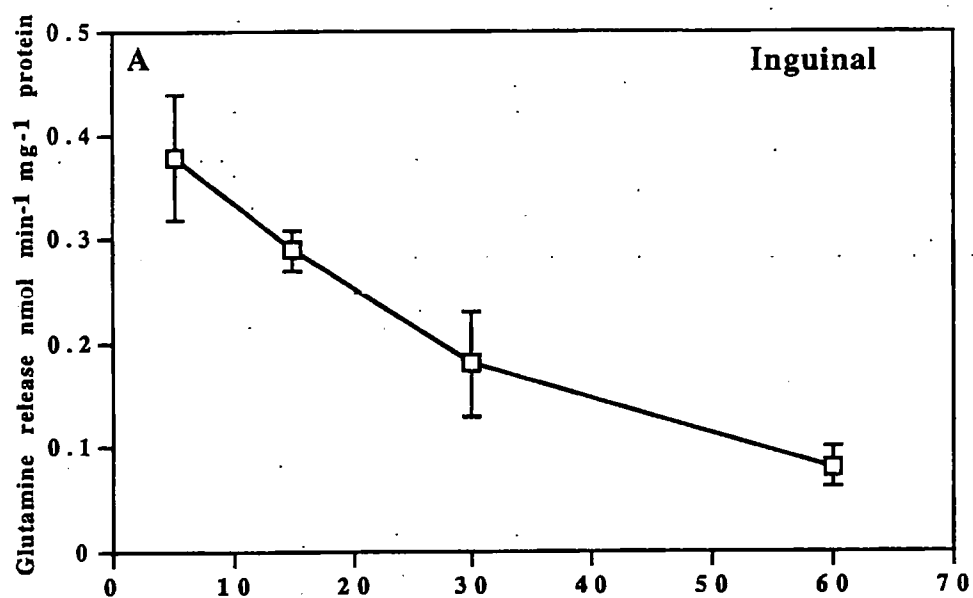
The rates of glutamine release from isolated adipocytes into the incubation media are expressed as $\text{nmol min}^{-1} \text{mg}^{-1}$ protein. Statistical analysis was carried out using Student's t-test. Differences in glutamine release were tested for significance between control and treated (insulin, dexamethasone or isoprenaline) samples by repeated-measures ANOVA.

5.3. Results

5.3.1. Glutamine release from isolated adipocytes over time

The rate of glutamine release from adipocytes was non-linear over a time period of 15 minutes in adipocytes prepared from all sites, with the release of glutamine being greatest when measured at 5 minutes of incubation time (Figures 5.1 and 5.2). The rate of change of glutamine release from adipocytes after 30 minutes of incubation time was not different from that measured after 60 minutes in all cases. An incubation time of 30 minutes was therefore chosen to study the effects of different hormones on glutamine release from isolated adipocytes prepared from different sites.

The data show some site-specific differences between sites in the relative amounts of glutamine released over time. Glutamine release from adipocytes isolated from the omental site was approximately twice that of all other sites measured over the 60 minute time course (Figure 5.1). There were no significant differences between the total values of glutamine release over 60 minutes of incubation time in adipocytes isolated from the other sites studied.



Time (minutes)

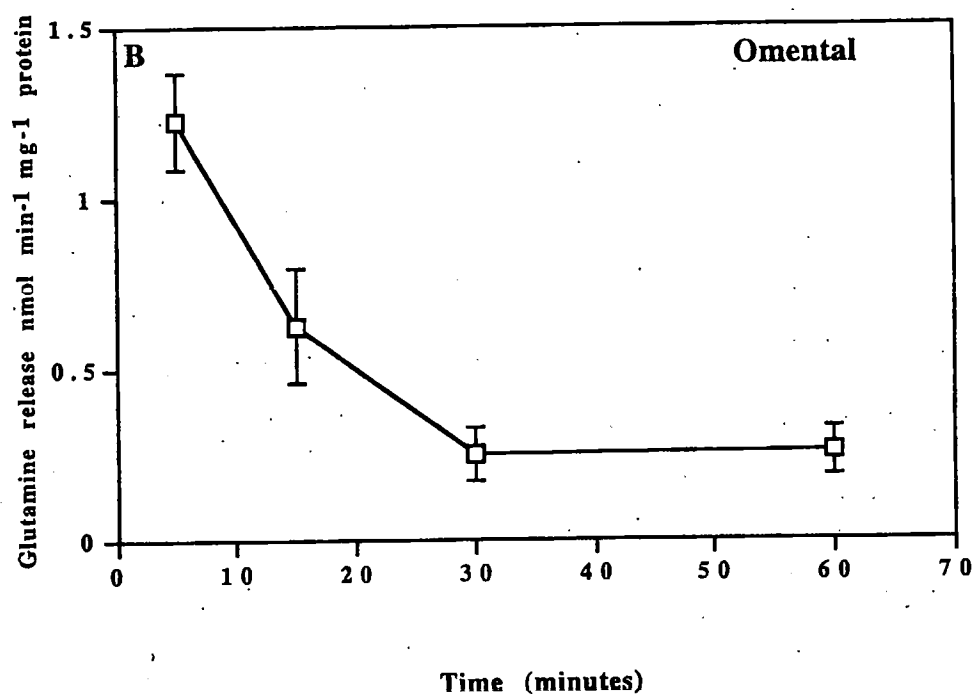
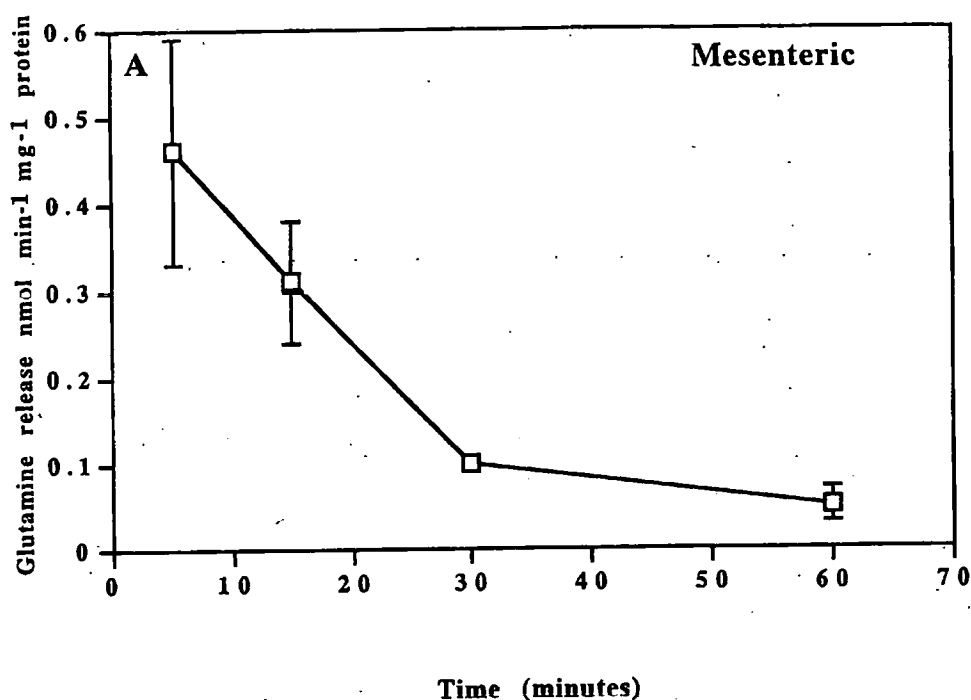


Figure 5.2 Glutamine release from adipocytes prepared from mesenteric and omental sites over a time course of 60 minutes, values are mean (\pm SEM) expressed as glutamine released into the incubation medium, nmol min⁻¹ mg⁻¹ protein from isolated adipocytes (n=4). (Note differences in y axis scales between graph A and B).

5.3.2. The effect of the branch-chain amino acid, leucine, on glutamine release from adipocytes prepared from five different adipose sites over a time course of 60 minutes

The addition of leucine to the incubation medium significantly enhanced glutamine release from adipocytes isolated from all sites studied after 5 minutes of incubation time (Table 5.1). The data show that adipocytes isolated from the omental site incubated for 5 minutes in the presence of leucine released approximately 1.5-fold that of adipocytes isolated from the same site incubated for 5 minutes in the absence of leucine (Table 5.1). Adipocytes isolated from inguinal, mesenteric and popliteal sites incubated for 5 minutes in the presence of leucine released approximately 2-fold more than paired samples of adipocytes incubated for 5 minutes in the absence of leucine (Table 5.1).

The greatest increase in glutamine release with leucine added to the incubation medium was observed in adipocytes isolated from the epididymal site. After 5, 15 and 30 minutes, epididymal adipocytes incubated with leucine released approximately 3.5, 3, and 2.5 times respectively more than adipocytes from the same site incubated in the absence of leucine (Table 5.1).

Site	5 minutes	15 minutes	30 minutes	60 minutes
Inguinal:				
Control	0.38 ± .06	0.29 ± .02	0.18 ± .05	0.11 ± .02
Leucine	0.64 ± .13**	0.34 ± .02	0.27 ± .03	0.13 ± .04
Mesenteric:				
Control	0.46 ± .13	0.31 ± .07	0.10 ± .01	0.08 ± .02
Leucine	0.81 ± .12***	0.27 ± .07	0.19 ± .02	0.05 ± .006
Omental:				
Control	1.23 ± .14	0.63 ± .10	0.25 ± .08	0.26 ± .07
Leucine	1.74 ± .14**	0.64 ± .01	0.26 ± .09	0.27 ± .06
Popliteal:				
Control	0.34 ± .09	0.25 ± .08	0.14 ± .03	0.12 ± .01
Leucine	0.59 ± .14*	0.39 ± .08	0.16 ± .04	0.11 ± .01
Epididymal:				
Control	0.41 ± .08	0.25 ± .04	0.16 ± 0.01	0.14 ± .01
Leucine	1.74 ± .16***	1.13 ± .14***	0.58 ± .11*	0.24 ± .09

Table 5.1 Glutamine release over a time course of 60 minutes with or without the addition of leucine to the incubation medium. Values are Mean (± SEM) expressed as glutamine released into the incubation medium, nmol min⁻¹ mg⁻¹ protein from isolated adipocytes (n=4). Statistical significance between the values of glutamine released from adipocytes incubated without leucine compared to those from the same site incubated with leucine at each time point are denoted by * P < 0.05, ** P < 0.01, *** P<0.001.

5.3.3. The effect of insulin on glutamine release from adipocytes prepared from five different adipose sites

Values of glutamine released from adipocytes were similar between all sites without the addition of insulin (between 0.16 and 0.26 nmol min⁻¹ mg⁻¹ protein). There was a 1.5 fold increase in the rate of glutamine release with insulin (50 mU ml⁻¹) in adipocytes isolated from the inguinal site. Insulin had no significant effect on the rate of glutamine release from adipocytes prepared from any of the other sites (Table 5.2).

5.3.4. The effect of dexamethasone and insulin plus dexamethasone on glutamine release from adipocytes prepared from five different adipose sites

In the presence of dexamethasone (2.5 mM), there was a 2.5-fold increase in the rate of glutamine release by adipocytes isolated from inguinal, omental and epididymal sites (Table 5.2).

The addition of insulin and dexamethasone to the incubation media resulted in a reduction in the magnitude of increase in glutamine release observed in adipocytes incubated with dexamethasone alone from isolated omental and epididymal adipocytes (Table 5.2).

5.3.5. The effect of isoprenaline on glutamine release from adipocytes prepared from five different adipose sites

Glutamine release was not significantly changed with isoprenaline in adipocytes isolated from the inguinal site (Table 5.2).

However, there was between a 60 and 70% decrease in glutamine release with isoprenaline (1 µM) from adipocytes isolated from the omental, mesenteric, epididymal and popliteal sites (Table 5.2).

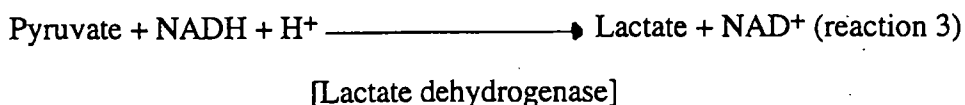
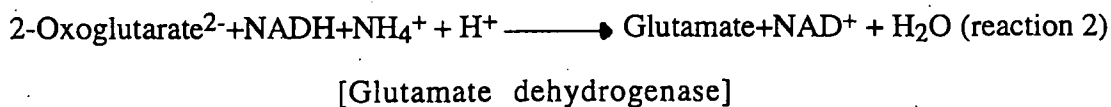
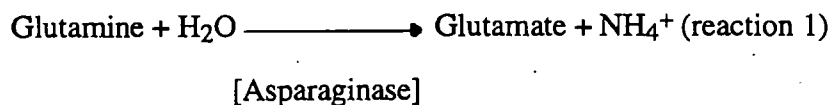
Depot	Control	Insulin	Dex.	Insulin + dex	Isoprenaline
Inguinal	0.16 ±.02	0.28 ±.03*	0.37 ±.02**	0.31 ±.05**	0.21±.03
Mesenteric	0.22 ±.02	0.25 ±.01	0.20 ±.05	0.17 ±.05	0.13 ±.01**
Omental	0.26 ±.03	0.21±.07	0.33 ±.02**	0.26 ±.02#	0.15 ±.07*
Popliteal	0.23 ±.02	0.19 ±.01	0.18 ±.01	0.17 ±.01	0.14 ±.03*
Epididymal	0.20 ±.03	0.21±.02	0.32±.06*	0.21±.04#	0.14 ±.02*

Table 5.2 Glutamine release by isolated adipocytes after 30 minutes of incubation in the absence (control) or presence of: insulin (50 $\mu\text{U ml}^{-1}$), dexamethasone (dex., 2.5 μM), insulin (50 $\mu\text{U ml}^{-1}$) with dex. (2.5 μM), and isoprenaline (1.0 μM). Values are mean (\pm SEM) expressed as glutamine released into the incubation medium, $\text{nmol min}^{-1} \text{mg}^{-1}$ protein from isolated adipocytes (n=4). Statistical significance between the values of glutamine released from adipocytes from different sites compared to those from the same site incubated without the addition of hormones are denoted by * $P < 0.05$, ** $P < 0.01$. Significant differences between adipocytes incubated with dex. or dex. plus insulin are denoted # $P < 0.05$.

5.4. Discussion

5.4.1. Investigation of glutamine release from isolated adipocytes prepared from different adipose sites over a time course of 60 minutes

The rate of glutamine release from isolated adipocytes in all sites measured decreased over time. Glutamine release from incubated skeletal muscle preparations also show a similar pattern of decline over time (Garber *et al.* 1976a, Parry-Billings 1989, Opara 1993). Garber *et al.* also reported a linear rate of glucose uptake and concluded that the reduction in the rate of glutamine uptake was not due to reduced viability of the skeletal muscle preparation. This study also showed, by performing experiments in which the incubation medium was changed periodically during the incubation period, that the decrease in the rate of glutamine release over time was not due to an accumulation of glutamine in the incubation medium. In the study by Opara (1993) it was concluded that the non-linear release of glutamine from skeletal muscle was probably caused by a 'carry over' effect of pyruvate from the pre-incubation media to the incubation media via muscle which interfered with the glutamine assay used to determine the concentration of glutamine in the incubation media. The concentration of glutamine in the media determined in the above studies for muscle glutamine release and in the present study involved the following reactions:



The concentration of glutamine in the incubation media was determined by measuring the conversion of NADH to NAD that occurs in reaction 2. It is possible that pyruvate may also react with NADH to give lactate and NAD^+ shown in reaction 3. This reaction is catalysed by lactate dehydrogenase released either from the adipocytes or contained as an impurity in the asparaginase or glutamate dehydrogenase. However, over an incubation of 60 minutes, the concentration of pyruvate is decreased through the action of lactate dehydrogenase to form lactate thereby lessening the effect of pyruvate on the glutamine assay.

In the present study, cell viability in the different adipocyte preparations may have affected the rate of glutamine release over time. However, at the end of the incubation period, all of the cell preparations excluded trypan blue and were therefore still viable. As the present study used same conditions as by Opara (1993), it is probable that the apparent decline in glutamine release was caused by an artefact in the carry-over effect of pyruvate, described above.

Site-specific differences in glutamine release over time were confined to adipocytes isolated from the intra-abdominal omental site which released approximately twice as much glutamine as adipocytes isolated from mesenteric, inguinal, epididymal and popliteal sites. The 2-fold increase in glutamine release in adipocytes isolated from the omental site correlates with the higher glutamine synthetase activity per g wet weight observed in adipose tissue extracts from this site in the fasted animals compared with that of all other sites (Chapter 4 Section 4.4.2). All of the other sites released glutamine at a similar rate over this time course. Therefore, although there were some minor site-specific differences in glutamine synthetase activity in adipose tissue extracts from fasted animals, this factor may not ultimately be the sole determinant of total glutamine release from these sites.

5.4.2. The effect of leucine on glutamine release from adipocytes prepared from five different adipose sites

Glutamine release was increased with the addition of leucine in adipocytes isolated from all sites after 5 minutes incubation compared to that of adipocytes incubated for the same time period without leucine. This difference was maintained for up to 30 minutes of incubation time in adipocytes isolated from the epididymal site only. The findings of the present study are in agreement with those of Tischler & Golberg (1980) who measured the effect of leucine on glutamine release *in vitro* from whole epididymal adipose tissue of fasted and fed rats. The study by Tischler & Goldberg found that the rate of glutamine release was increased with leucine in epididymal adipose tissue from both the fed and the fasted rats and was higher in adipose tissue from the fed rats compared to that of the fasted animals. Tischler & Goldberg concluded that the ability of leucine to enhance glutamine release probably resulted from its *de novo* synthesis from this precursor and not through promotion of protein degradation or from liberation of existing intra-cellular glutamine pools.

5.4.3. Glutamine release from adipocytes prepared from different adipose sites in the presence of insulin compared to the activity of glutamine synthetase in adipose tissue extracts with feeding

The addition of insulin to the incubation medium resulted in a 1.5-fold increase in the rate of glutamine release from adipocytes isolated from the inguinal site but not from adipocytes isolated from the other sites studied. Glutamine synthetase activity was increased by approximately 2-fold (per g wet weight and per mg protein) in adipose tissue extracts from the inguinal site directly after feeding compared to that of the fasted animals. However, there were no significant differences in glutamine synthetase activity after feeding in extracts of adipose tissue from any of the intra-abdominal sites (Chapter 4 Section 4.4.3). The lack of effect of insulin on glutamine release from mesenteric and omental adipocytes combined with the relatively unchanged activity of glutamine synthetase after feeding in adipose tissue extracts from these sites suggest that glutamine

release after a meal would not be significantly altered in these sites. The 2-fold increase in glutamine synthetase in extracts of inguinal adipose tissue directly after feeding closely correlates with the 1.5-fold increase in glutamine release from isolated inguinal adipocytes. Furthermore, after feeding, the total estimated '*in vivo*' glutamine synthetase activity for this depot was significantly higher than that of all the remaining adipose depots directly following a meal (Chapter 4, Section 4.5.5). The inguinal adipose depot in adult guinea-pigs represents approximately 16% of total adipose tissue mass and from the '*in vivo*' calculations, has the capacity to contribute to approximately 20% of the total activity of glutamine synthetase (Table 4.3, Chapter 4). Therefore, it is likely that this depot releases significant quantities of glutamine into the circulation, particularly in the fed state. These findings are in agreement with the study by Kowalski & Watford (1994a) in which glutamine release from inguinal adipose tissue of *ad lib* fed rats was measured *in vivo* using a microdialysis sampling technique. Kowalski & Watford estimated that this site released approximately twice as much per g wet weight compared with that estimated for rat skeletal muscle from data reported by Ardawi & Jamal (1990).

Although glutamine release was not measured in adipocytes isolated from any of the remaining superficial sites or the perirenal depot, it is possible that release may also be enhanced in the presence of insulin in these depots as an increase in glutamine synthetase activity occurred in this depot directly after feeding. Therefore, a significant proportion (approximately 50%) of the total adipose tissue mass may contribute to the circulating glutamine pool in the early post-prandial period.

The mechanism by which insulin may enhance glutamine synthesis and release in adipocytes is unknown. In adipose tissue, glutamine could be synthesized from α -ketoglutarate, which can be formed from glucose or pyruvate. The availability of these substrates is increased in the early post-prandial period, along with that of other amino acids including BCAA (leucine, isoleucine and valine) (Elia *et al.* 1989). It is thought that BCAA are the main substrates for glutamine synthesis in adipose tissue, the amino groups from leucine deamination in particular, are released as α -amino nitrogen in glutamine (Tischler and Goldberg 1980). In the previous study, (Section 5.4.2.) the presence of

leucine in the incubation medium enhanced glutamine release from adipocytes isolated from all five adipose sites. It is therefore possible that in some way insulin enhances the intracellular metabolism of amino acids leading to the synthesis and release of glutamine from adipose tissue.

5.4.4. Glutamine release from adipocytes prepared from different adipose sites in the presence of dexamethasone compared to the activity of glutamine synthetase in adipose tissue extracts

Glutamine release was enhanced in the presence of dexamethasone in adipocytes isolated from the inguinal, omental and epididymal sites. These findings are in agreement with previous reports that glutamine release *in vitro* is increased from epididymal adipose tissue of rats treated for nine days with dexamethasone (Ardawi and Jamal 1990a). Furthermore, glutamine synthetase activity has been shown to be significantly increased in epididymal and perirenal adipose tissue of dexamethasone treated rats (Opara 1993).

In the fasted state, serum cortisol levels were significantly greater than those of the fed animals (Chapter 3, Section 3.4.5). Furthermore, there was measurable glutamine synthetase activity after an 18 hour overnight fast in adipose tissue extracts from all sites. It is therefore possible that the relatively high circulating cortisol levels observed with fasting serve to maintain the synthesis and release of glutamine from adipose tissue, with some depots, such as inguinal, omental and epididymal, contributing more than others to the circulating glutamine pool. In humans, muscle has been demonstrated to release significant amounts of glutamine into the circulation and this is taken up by the splanchnic bed in the post-absorptive state (Marliss *et al.* 1971) With fasting, it has been suggested that glutamine serves as an important hepatic and renal gluconeogenic precursor in humans (Nurjhan *et al.* 1995 and Perriello *et al.* 1997). In the postabsorptive rat, glutamine released from muscle does not account for the total amount utilised by the splanchnic bed (Welbourne 1987). Therefore, the capacity of certain adipose tissue depots to synthesize and release glutamine in the post-absorptive state provides an additional supply of this amino acid.

The addition of insulin as well as dexamethasone to the incubation medium resulted in values of glutamine release falling to that of the control values in isolated omental and epididymal adipocytes compared to that of adipocytes from the same site treated with dexamethasone only. The reasons for this observed reduction in glutamine release are not clear. Incubation of 3T3-L1 adipocytes with insulin results in a significant increase in glutamine synthetase activity and protein synthesis (Miller *et al.* 1978). It is possible therefore that the combined action of these hormones in isolated adipocytes from certain adipose sites serves to divert intra-cellular glutamine away from release and into protein synthesis.

5.4.5. Glutamine release from adipocytes prepared from different adipose sites in the presence of isoprenaline compared to the activity of glutamine synthetase in adipose tissue extracts with exercise

Incubation with isoprenaline resulted in a decrease in glutamine release in isolated adipocytes prepared from mesenteric, omental and popliteal sites. A similar effect has been reported to occur when physiological concentrations of adrenaline, noradrenaline or isoprenaline are added to rat skeletal muscle preparations *in vitro* (Garber *et al.* 1976b). In the same study, the tissue levels of glutamine were also reduced after 60 minutes incubation with epinephrine and dbcAMP, whereas in control preparations, glutamine levels were unchanged. The study by Garber *et al.* (1976b) concluded that the reduction in glutamine release was a result of reduced net amino acid synthesis from muscle proteolysis, thus conserving muscle protein and limiting the supply of precursors for glutamine synthesis. The effects of isoprenaline on adipocyte glutamine metabolism have not to the author's knowledge been reported. However, as discussed in the previous chapter on glutamine synthetase activity, 3T3-L1 adipocytes incubated with theophylline and dbcAMP demonstrate a rapid decline in glutamine synthetase biosynthesis, and activity (Miller and Burns 1985). In the study described in Chapter 4, Section 4.5.4., glutamine synthetase activity was significantly reduced in adipose tissue extracts from the intra-abdominal omental, mesenteric and epididymal sites compared to that of the fasted

animals. Enzyme activity was also reduced in adipose tissue extracts from the popliteal site but these values did not reach statistical significance, due to the relatively high standard errors of the mean.

The site-specific reduction in glutamine release with isoprenaline in isolated adipocytes, coupled to the concomitant reduction in glutamine synthetase activity directly after exercise (Chapter 4, Section 4.5.4), suggests that the intra-abdominal sites, and possibly the intermuscular popliteal site, reduce their capacity to both synthesise and release glutamine with exercise when local concentrations of the catecholamines are elevated.

In guinea-pigs, adipose tissue from the intermuscular sites in particular has been shown to be more sensitive than that of the larger depots (inguinal and perirenal) to the lipolytic effects of noradrenaline *in vitro* (Pond and Mattacks 1991). Furthermore, in rats, noradrenaline-induced lipolysis has been reported to be much greater in isolated adipocytes prepared from the intra-abdominal omental and epididymal sites than that of the subcutaneous inguinal site (Morimoto *et al.* 1997). Under conditions of exercise or stress, where the demand for lipid by muscle is increased, fatty acids are preferentially mobilised from the intra-abdominal depots into the portal circulation, and from the subcutaneous in front of fore-arm depot and the intermuscular popliteal depot possibly for use by adjacent skeletal muscle. The results from the present study and those presented in Chapter 4 indicate that under similar conditions these sites reduce the capacity to synthesise and release glutamine. However, the large subcutaneous sites such as inguinal remain relatively unaffected and maintain the capacity to synthesise and release glutamine. The data from this study suggests that this relatively large depot would therefore maintain a significant capacity to contribute to circulating glutamine.

5.4.6. Conclusion

Glutamine release over time was higher in adipocytes from the omental site than that from the remaining sites over time. The combination of a high glutamine release and a relatively high glutamine synthetase activity in this site indicates that the greater omentum has a

significant capacity to contribute both to adjacent lymphoid tissue and to the portal supply of glutamine.

The addition of insulin, dexamethasone or isoprenaline had site-specific effects on the rate of glutamine release in isolated adipocytes prepared from different adipose sites. Insulin increased the rate of glutamine release from isolated adipocytes prepared from the inguinal site only. As glutamine synthetase activity was also increased after feeding in this large subcutaneous depot, it is likely that it contributes significantly to circulating glutamine in the fed state.

Dexamethasone enhanced glutamine release in isolated adipocytes prepared from inguinal, omental and epididymal sites. In physiological conditions such as fasting, when circulating cortisol levels are elevated, the synthesis and release of glutamine from certain large adipose depots would serve to provide an additional supply, along with muscle, of glutamine for gluconeogenesis.

The combination of dexamethasone with insulin caused a reduction in the release of glutamine from isolated adipocytes prepared from omental and epididymal sites. In the present study, glutamine synthetase activity was not studied in any physiological condition where the circulating concentrations of these two hormones would be expected to be concomitantly elevated, such as sepsis. Therefore, it is difficult to assess whether glutamine synthesis may also be affected by insulin and cortisol *in vivo*. However, the apparent reduction in glutamine release from these two sites could lead to the speculation that proteolysis would be inhibited and protein synthesis would be promoted.

There was also a site-specific effect of isoprenaline on glutamine release in adipocytes prepared from two intra-abdominal sites and one intermuscular site. Lipolysis in these sites is known to be highly responsive to the catecholamines and in this study, showed that they respond to the beta agonist isoprenaline by reducing glutamine release, therefore the combined effect of catecholamines *in vivo* could act to conserve protein and favour lipid release.

The data presented in this study and in the previous study in Chapter 4 demonstrate site-specific differences in the capacity of adipose tissue to synthesise and

release glutamine in response to different hormones (or their analogues) which may regulate the flow of this amino acid to other utilising tissues and organs with altering physiological conditions.

Chapter 6

Discussion

6.1. Introduction

It is well established that glutamine, as well as serving as a major carrier of nitrogen between tissues, plays a central role in many metabolic and biosynthetic pathways in different tissues and organs. During the post-absorptive period, the small intestine is a major utiliser of glutamine in humans and rodents, extracting between 30 and 40% of the total plasma glutamine in each blood pass through the tissue (Windmueller 1982). In the post-prandial period, the small intestine continues to utilise plasma as well as dietary glutamine. Despite the considerable utilisation of glutamine by the small intestine, plasma glutamine concentrations in post-absorptive and post-prandial humans remain relatively unchanged (Elia *et al.* 1989), indicating the synthesis and release of this amino acid from other tissues and organs is closely regulated to maintain a steady supply with altering physiological conditions.

The role played by muscle in the provision of glutamine has been well described and its capacity to synthesise and release this amino acid varies according to physiological status (King *et al.* 1983; Willhoft *et al.* 1993; Falduto *et al.* 1992a and b). However, calculations for whole-body glutamine balance in the rat show that glutamine production by muscle alone does not account for the net utilisation by the kidney, small intestine and immune system (Shrock and Goldstein 1981; Welbourne 1987).

Although it has long been known that adipose tissue has the capacity to both utilise and release glutamine, its role in the context of whole-body metabolism of this important amino acid is far from clear. Frayn *et al.* (1991) using arteriovenous techniques showed that before and after a meal, human superficial abdominal adipose tissue takes up glutamate and releases glutamine. Assuming that all depots behave in the same way, adipose tissue may contribute to as much as one third of that of skeletal muscle to whole-body glutamine production. Kowalski and Watford (1994a) using a microdialysis sampling technique reported similar findings in rat inguinal adipose tissue and calculated that whole-body glutamine production may almost equal that of muscle. Limitations of

these studies were that the results were obtained from measurements of arteriovenous differences in glutamine and glutamate and simultaneous uptake and release of glutamine could not be accounted for. In addition, the estimated fluxes of glutamine and glutamate were measured in only one adipose depot, thus precluding possibility of site-specific differences in the metabolism of glutamine which could affect the overall estimations of adipose tissue's contribution to whole-body glutamine supply.

The guinea-pig model used in this study of glutamine metabolism was not ideal as they are natural grazers used to a diet consisting of mainly carbohydrate. A similar study using omnivorous rats may possibly have shown a greater capacity for adipose tissue to metabolise glutamine, but would not have given as much information about site-specific differences in glutamine metabolism because rats do not have sufficient adipose tissue in all of the major adipose depots with which to perform such a detailed study. The data from the present study, however, show that guinea-pig adipose tissue has a significant capacity to metabolise glutamine through the action of glutaminase and glutamine synthetase (summarised in Table 6.1 and 6.2). Furthermore, site-specific differences in the activities of these enzymes after feeding, fasting and exercise were evident.

The physiological relevance of these findings alone do not give a complete picture of the metabolism of glutamine in different adipose tissue depots with feeding, fasting and exercise as removal of an enzyme from its natural environment may give a distorted view of its characteristics *in vivo*. Other factors such as substrate availability may also play an important role in determining the effect of alterations in glutaminase activity on the flux of glutamine in adipose tissue with feeding, fasting and exercise. A summary of site-specific differences in glutaminase and glutamine synthetase activity compared with alterations in glutamine uptake and release with different hormones or their analogues is shown in Tables 6.1 and 6.2.

Table 6.1 Summary of glutaminase activity in extracts of adipose tissue from different adipose depots and glutamine uptake with dexamethasone, insulin and isoprenaline in isolated adipocytes. Data taken from Table 2.3 and 2.4, Chapter 2 and Figures 3.2 to 3.6, Chapter 3.

DEPOT	Glutaminase (whole adipose tissue)				Glutamine Uptake (isolated adipocytes)	
	Fasted	Fed	Exercised	Dexamethasone	Insulin	Isoprenaline
Inguinal	High	2 fold increase	5 fold decrease	no change	2 fold increase (10 μ U ml ⁻¹)	1 fold decrease (100 nM)
Mesenteric	low	3 fold increase	no change	no change	2 fold increase (1000 μ U ml ⁻¹)	1.7 fold decrease (1000 nM)
Omental	low	5 fold increase	decrease	no change	2.5 fold increase (100 μ U ml ⁻¹)	decrease (1000 nM)
Epididymal	medium	1.7 fold increase	5 fold decrease	no change	2 fold increase (100 μ U ml ⁻¹)	no change
Popliteal	medium	2 fold increase	10 fold decrease	no change	2 fold increase (100 μ U ml ⁻¹)	no change

Table 6.2 Summary of glutamine synthetase activity in extracts of adipose tissue from different adipose depots and glutamine release dexamethasone, insulin and isoprenaline in isolated adipocytes. Data taken from Table 4.1 and 4.2, Chapter 4 and Table 5.2, Chapter 5.

DEPOT	Glutamine synthetase (whole adipose tissue)			Glutamine release (isolated adipocytes)		
	Fasted	Fed	Exercised	Dexamethasone (2.5 mM)	Insulin (50 μ U ml ⁻¹)	Isoprenaline (1 μ M)
Inguinal	High	2.5 fold increase	no change	2 fold increase	1.7 fold increase	no change
Mesenteric	low	no change	2 fold decrease	no change	no change	1.6 fold decrease
Omental	high	no change	2 fold decrease	1.2 fold increase	no change	1.7 fold decrease
Epididymal	medium	2 fold increase	3.7 fold decrease	no change	no change	1.4 fold decrease
Popliteal	medium	no change	no change	no change	no change	1.4 fold decrease

These Tables show that the studies of uptake and release of glutamine in viable isolated adipocytes prepared from different adipose sites in the presence of insulin, dexamethasone and isoprenaline, reported in Chapters 3 and 5, have shown some site-specific differences in the response to these hormones or their analogues which are consistent with the site-specific alterations in glutaminase and glutamine synthetase after feeding fasting and exercise (Chapter 2 and 4).

6.2 Glutaminase activity in adipose tissue extracts and glutamine uptake in isolated adipocytes prepared from different adipose depots

A summary of the changes in glutaminase activity measured in adipose tissue extracts from five different adipose depots of fed, fasted and exercised guinea-pigs is shown together with the effect of insulin, isoprenaline and dexamethasone in isolated adipocytes prepared from the same sites in Table 6.1.

In the present study, there were significant site-specific alterations in glutaminase activity assayed under optimal conditions in adipose tissue extracts after fasting, feeding and exercise (Chapter 2, Sections 2.4.7 to 2.4.10). With fasting, the greatest enzyme activity was observed in adipose tissue extracts from the omental (adjacent to spleen) and inguinal samples indicating that these sites may maintain a significant capacity to hydrolyse glutamine in the fasted state (Chapter 2, Table 2.3 and Figure 2.4). Glutaminase activity measured in the remaining sites studied were relatively low and not significantly different from one another.

In the early post-prandial period, glutaminase activity was higher in adipose tissue extracts from all sites studied compared to that of the fasted values with samples from the cervical and omental (far from spleen) sites demonstrating the greatest increases in enzyme activity. However, at 60 minutes after a meal, glutaminase activity was not detectable in any site except the omental (adjacent to spleen) site. At 240 minutes after feeding glutaminase activity was again measurable in adipose tissue extracts from all sites studied (Chapter 2, Table 2.3 and Figure 2.4). These time-dependent alterations in glutaminase activity suggest that early on in the post-prandial period, adipose tissue increases its

capacity to hydrolyse glutamine. Over the post-prandial time-course used in the present study, circulating amino acids along with glucose absorbed from the diet would be expected to be higher at 30 minutes, with chylomicron-associated triacylglycerol appearing in the circulation at between 60 and 240 minutes after a meal (Elia *et al.* 1989; Coppack *et al.* 1990). The post-prandial increase in glutaminase activity measured in adipose tissue extracts at 30 minutes after feeding therefore correlates with the expected rise in the circulating concentration of glutamine and its subsequent availability to adipose tissue. At 60 minutes after a meal, glutaminase activity *in vitro* was decreased to below the levels of detection and occurs at a time when the availability of lipoprotein-bound triacylglycerol to adipose tissue is increased. This observed disappearance of enzyme activity at 60 minutes would therefore allow adipose tissue to deal primarily with the metabolism of fatty acids, with glutamine metabolism taking second place. There is some evidence that the early post-prandial metabolism of glutamine may also have an additional effect on lipolysis in adipose tissue. Previous studies have shown that an infusion of glutamine in fasted dogs (Cersosimo *et al.* 1986) and in humans results in an inhibition of lipolysis (Déchelotte *et al.* 1991). The latter study proposed that an increase in the hydrolysis of glutamine in the adipocyte would result in accumulation of glutamate which could shift the redox potential from NADH to NAD and from NADPH to NADP thus favouring lipogenesis and inhibiting lipolysis.

After exercise, glutaminase activity was significantly reduced compared to the fasted values in adipose tissue extracts from the omental (far from spleen), epididymal, perirenal and popliteal sites (Chapter 2, Table 2.5). Such alterations in glutaminase activity would therefore serve to reduce the breakdown of glutamine in adipose tissue. Furthermore, a reduction in the accumulation of glutamate within the adipocyte may also serve to reverse its anti-lipolytic effect, and, together with sympathetic nervous stimulation, enhance lipolysis.

The study described in Chapter 3 addresses whether uptake of glutamine into viable adipocytes *in vitro* is altered by hormones likely to be present in the circulation with feeding, fasting and exercise. Glutamine uptake into isolated adipocytes in the presence of

insulin, dexamethasone or isoprenaline was measured in order to establish whether the intracellular availability of this amino acid in adipose tissue may be altered after a meal or after brief, acute exercise. Serum cortisol levels were found to be higher in the fasted animals than in the post-prandial animals. However, uptake of glutamine into isolated adipocytes prepared from all sites studied was unaffected by the addition of a glucocorticoid analogue, dexamethasone. These data indicate that in the morning pre-prandial state when circulating glucocorticoids are elevated adipose tissue may not participate significantly in the removal of circulating glutamine.

A physiological concentration of insulin of between 10 and 100 $\mu\text{U ml}^{-1}$ was demonstrated to stimulate glutamine uptake into isolated adipocytes (Chapter 3. Section 3.4.2). The greatest response to insulin was observed in isolated adipocytes prepared from the inguinal and omental (far from spleen) sites. In adipocytes isolated from the popliteal and mesenteric sites, there was a weak response to insulin but a concentration outside the physiological range (1000 $\mu\text{U ml}^{-1}$) was required to achieve a two fold increase in glutamine uptake. The enhanced uptake of glutamine in the presence of physiological concentrations of insulin coupled to the observed "*ex vivo*" increases in glutaminase activity in the early post-prandial period indicate that the relatively large adipose depots, inguinal and omental, have a significant capacity to contribute to the disposal of dietary glutamine in the early post-prandial period. In the present study, the fate of glutamine in the adipocyte was not assessed, but a previous study by Kowalchuk *et al.* (1988) demonstrated that rat epididymal isolated adipocytes utilise glutamine with the incorporation of its carbon into triacylglycerols when insulin (10 mU ml^{-1}) and glucose (1 mM) are included in the incubation medium. In the present study, the effect of insulin on glutamine uptake in isolated adipocytes and the concomitant rise in glutaminase activity in adipose tissue extracts in the early post-prandial period indicate that the omental and inguinal depots have a high capacity to utilise the glutamine carbons, possibly for triacylglycerol synthesis. These two large adipose depots in guinea-pigs and rats represent approximately 30% of the total adipose tissue mass and could therefore utilise glutamine to synthesise significant amounts of triacylglycerols. It is not known whether site-specific

differences in glutamine metabolism contribute to the distribution and accumulation of adipose tissue lipids in mammals, however, in those which consume a very low protein diet, for example ruminants, especially Camelidae, the size of the inguinal depot in particular, is relatively small compared to that of carnivores whose diet consists mainly of protein (reviewed by Pond 1992). Humans have pronounced sex differences in adipose tissue distribution, characterised by selective accumulation of adipose tissue in the femoral and gluteal regions in pre-menopausal women. However, the metabolic reasons for sex-differences in adipose tissue distribution remain unclear, despite intensive efforts to demonstrate that aspects of lipid metabolism are responsible, such as increased lipoprotein lipase activity in adipose tissue from the femoral/inguinal (Rebuffé-Scrive *et al.* 1987). To the author's knowledge, no studies on sex-specific differences in adipose tissue glutamine metabolism have been performed, and it would be interesting to ascertain how such differences, if they exist, may contribute to the sex-specific distribution of adipose tissue in humans.

The results from the present study also indicate that lipogenesis may be enhanced by an increase in glutamine metabolism in the intra-abdominal omental depot. In humans, this depot is subject to hypertrophy, particularly in middle aged males. Almost all previous attempts to explain these observations have related to lipid metabolism but to the author's knowledge there have been no studies to date on glutamine metabolism in human intra-abdominal adipose tissue. The western diet, as well as containing a high proportion of lipid, also contains a substantial amount of animal protein, approximately 70-100 g per day often consumed in one or two meals. The effect of a relatively high protein intake on human intra-abdominal adipose tissue metabolism and accumulation has not been studied. The significant capacity for glutamine metabolism shown by this study in guinea-pigs warrants further investigation of glutamine metabolism in humans to establish whether an excess of dietary amino acids may contribute to central adiposity in middle age.

Incubation with isoprenaline of isolated adipocytes prepared from the inguinal, mesenteric and omental (far from spleen) sites (Table 6.1) resulted in decrease in glutamine uptake. This reduction in glutamine uptake in certain adipose sites such as the

inguinal and omental sites (which have a significant capacity to utilise glutamine in the fasted and fed state), coupled to the observed reduction in glutaminase activity with exercise, would serve to further limit its metabolism when catabolic conditions prevail and facilitate the restoration of glutamine levels in the circulation whence it can be taken up by other tissues such as muscle and the intestine.

6.3 Glutamine synthetase activity in adipose tissue extracts and glutamine release in isolated adipocytes prepared from different adipose depots

The study of glutamine synthetase activity in adipose tissue extracts demonstrates that adipose tissue from all sites examined maintains the capacity to synthesise glutamine in the fasted state. Quantitative analysis of enzyme activity by estimating *in vivo* activity showed that the relatively large interscapular, inguinal, and perirenal depots and the smaller omental (adjacent to spleen) site contribute most, and the mesenteric least, to the synthesis of glutamine during fasting. Directly after feeding, glutamine synthetase activity was increased in adipose tissue extracts from the four large superficial sites, in front forelimb, behind forelimb, interscapular and inguinal, in the intra-abdominal epididymal and the intermuscular popliteal and cervical sites. Values of enzyme activity measured in the interscapular, inguinal, cervical and popliteal sites remained significantly greater than those measured in adipose tissue from the fasted group for up to 240 minutes after a meal. The estimated '*in vivo*' activity of glutamine synthetase showed that adipose tissue from all depots except mesenteric increases its capacity to synthesise glutamine for up to 240 minutes after feeding. The estimated values of '*in vivo*' glutamine synthetase activity are comparable to the values of *in vivo* glutamine release from inguinal adipose tissue of *ad lib.* fed rats (Kowalski and Watford 1994a) and with *in vitro* release from rat epididymal adipose tissue (Ardawi 1988). To the author's knowledge, there have been no previous studies on the process of glutamine synthesis and release in adipose tissue so it is not known whether alterations in glutamine synthetase activity are synonymous with changes in glutamine release.

In muscle, it is thought that glutamine synthesis and glutamine release are independent processes and that glutamine release rather than glutamine synthesis is the flux-generating step (Newsholme and Parry-Billings 1990); it was identified as the first non-equilibrium reaction that approaches saturation with the pathway substrate and there is evidence that the flux generating step is the process of glutamine release from muscle. It was further suggested that the rate of synthesis of glutamine in muscle normally has no effect on the rate of glutamine release from muscle and that changes in glutamine synthetase activity serve primarily to maintain a steady-state concentration of glutamine in muscle. Glutamine release from muscle may be externally regulated by alterations in cell volume and/or differences in efficiency of intra-cellular transporters which translocate glutamine from intracellular compartments to the sarcolemma.

The results of the present study do not show quantitatively how adipocytes may contribute to the levels of circulating glutamine, as they have been removed from their natural environment which almost certainly contains other factors that may affect the release of this amino acid *in vivo*. However, the response of isolated adipocytes prepared from different adipose sites in the absence or presence of the BCAA leucine, and of hormones (or their analogues) coupled to the site-specific alterations in glutamine synthetase activity after feeding, fasting and exercise gives qualitative information on how different adipose sites alter their ability to contribute to local and whole-body glutamine supply.

In the present study, isolated adipocytes prepared from five different adipose sites incubated in KRB supplemented with glucose released glutamine into the incubation medium over a time course of 60 minutes. Adipocytes from the omental site (far from spleen) released twice as much glutamine as that of adipocytes isolated from the remaining sites. The relatively high rate of glutamine release from omental adipocytes correlates with the high activity of glutamine synthetase measured in adipose tissue extracts from this site in the fasted animals (Table 6.2).

The high capacity for omental adipose tissue to synthesise and release glutamine may serve to increase the glutamine concentration in the portal circulation. In addition,

release of glutamine from adipose tissue of the greater omentum may also supply glutamine locally. This depot characteristically contains a significant amount of lymphoid tissue, the greatest proportion of which are macrophages and the remainder consisting of B lymphocytes, T lymphocytes and mast cells (Shimotsuma *et al.* 1993). Lymphocytes have been shown to utilise glutamine at a high rate reviewed by (Calder 1994) and the phagocytic ability of macrophages *in vitro* is dependent upon the concentration of glutamine in which the cells have been cultured (Parry-Billings and Newsholme 1990). Significantly, in adipocytes isolated from the omentum glutamine release was enhanced in the presence of dexamethasone (Table 6.2). Elevated circulating glucocorticoid levels are known to be associated with an immune challenge and enhancement of glutamine release from adipose tissue in this region of the omentum may be of particular benefit to local lymphoid tissues. The high capacity of omental adipocytes to synthesise and release glutamine demonstrated by the present study warrants further investigation. Previous studies in which adipose tissue explants were co-incubated with lymphoid cells show that lipolysis in omental adipose tissue is strongly stimulated by secretions from immune cells (Pond and Mattacks 1995 and Mattacks and Pond 1997). It is also possible that glutamine release is also stimulated under such conditions and contribute to local interactions between omental adipose tissue and lymphoid cells. The enhanced ability of omental adipose tissue to supply glutamine may be utilised locally and therefore not necessarily contribute significantly *per se* to the circulating peripheral concentrations of this amino acid.

Glutamine release from isolated adipocytes prepared from inguinal and epididymal adipose tissue was also enhanced in the presence of dexamethasone (2.5 μ M) compared to that of the control values, whereas, this process was unaffected in adipocytes from the mesenteric and popliteal sites by the addition of dexamethasone (Table 6.2). These site-specific differences in the response of adipocytes to this glucocorticoid analogue suggest that the latter two sites may not contribute significantly to circulating glutamine levels when plasma cortisol levels are increased. Although glutamine release was not measured in adipocytes prepared from all adipose depots studied previously, it is

possible that glutamine release may also be enhanced in the remaining depots as they also were shown to possess a high capacity to synthesise glutamine through the activity of glutamine synthetase, which has been shown to be increased in samples of epididymal and perirenal adipose tissue from glucocorticoid-treated rats (Opara 1993). In the rat, these two sites do not contain any lymphoid tissue and hence glutamine may be released into the circulation for whole-body glutamine supply.

6.4 Activities of glutaminase and glutamine synthetase in adipose tissue extracts suggests substrate cycling of glutamine

The existence of a glutamine/glutamate substrate cycle in muscle has previously been proposed by Newsholme and Parry-Billings (1990) as glutamine is taken up and released by this tissue in separate processes which are both non-equilibrium. These workers suggested that the existence of such a cycle would serve to enhance the sensitivity of the mechanism(s) that control the rate of glutamine release from muscle. Alterations in the levels of hormones which affect the both the uptake and release of glutamine may serve to either increase or decrease the rate of cycling within muscle which in turn could increase or decrease the sensitivity of glutamine release to changes in the concentration of other regulators, including other hormones and/or cytokines. Consequentially, the rate of glutamine release from muscle may be altered in such a way as to control the supply of glutamine and maintain the plasma concentration of this amino acid.

In the present study, simultaneous activity of glutaminase and glutamine synthetase were measured in extracts of guinea-pig adipose tissue from all the major sites in the overnight-fasted state, directly after, and at 30 minutes after feeding (Chapter 2, Section 2.4 and Chapter 4, Section 4.4). Concomitant activation of these two enzymes coupled to the capacity for adipose tissue to take up and release glutamine (Chapter 3, Section 3.4 and Chapter 5, Section 5.4) indicates that this tissue can both utilise and synthesise glutamine simultaneously and suggests the existence of a substrate cycle between glutamine and glutamate in adipose tissue. The existence of such a substrate cycle in adipose tissue would enable it to utilise available glutamine efficiently for conversion

into triacylglycerol and to maintain circulating glutamine levels. This kind of substrate cycle also exists with fatty acids and triacylglycerols in adipose tissue and have been shown to have large site-specific differences that alter selectively after exercise and feeding (Mattacks and Pond 1988).

It is also important to consider that in guinea-pigs and in humans, the percentage of protein in whole adipose tissue is less than 1% of its mass, with 70-80% constituting lipid and the remainder as fluid. Only a very small proportion of the adipocyte mass could contribute significantly to glutamine supply, so concomitant activation of glutaminase and glutamine synthetase could allow substrate cycling preventing valuable amino acids from being 'lost' as lipid.

In addition, as adipose tissue mass and adipocyte volume declines in situations of weight loss, the inhibitory effect of lipids on amino acid metabolism (Chapter 2, Section 2.4.4) may be reduced to allow the process of amino acid metabolism in adipose tissue to proceed at a more rapid rate and serve as an additional important supply of circulating glutamine as well as that of muscle.

6.5 Conclusions

This study took a broad approach to the metabolism of glutamine in adipose tissue looking first at the site-specific changes in enzyme activity and then the uptake and release of glutamine in adipocytes. A detailed study of the flux of glutamine in adipose tissue from one or two depots may have revealed more detailed and quantitative information about the fate of glutamine in the adipocyte but would not have given such a broad physiological view of glutamine metabolism in adipose tissue mass as whole. This study points future workers towards sites that are more likely to provide relevant information about glutamine metabolism in adipose tissue, and how different sites may contribute more or less to whole-body and local glutamine supply. The larger depots, including omental and inguinal may contribute most per g tissue to local glutamine supply in the former and whole-body in the latter. The intermuscular popliteal site and mesenteric, appear to play little part in the

metabolism of glutamine, which contrasts with their high sensitivity to lipolytic and lipogenic agonists (Pond and Mattacks 1991).

The relative importance of adipose tissue's role in glutamine metabolism may be emphasised by considering physiological conditions associated with muscle wasting and loss of adipose tissue, observed with cachexia and anorexia and in the overtrained athlete. The burden to supply glutamine is believed to be placed mainly with skeletal muscle. As circulating glutamine levels decline rapidly in these situations, there is some evidence to show that muscle alone is not able fulfil the demand for glutamine from utilising tissues such as the intestine and the immune system, and suggests that the contribution of adipose tissue may be of importance in maintaining glutamine levels in the circulation. The quantitative role of adipose tissue in whole-body (and local) glutamine metabolism, and how glutamine metabolism in adipose tissue is regulated in healthy and disease states, has not been studied and requires continued investigation to understand the importance of this tissue's contribution to whole-body glutamine metabolism.

6.6. Future work

1. This study has shown site-specific differences in guinea-pig adipose tissue glutamine metabolism and points future workers to depots, such as inguinal and omental, which have a significant capacity to contribute to either whole-body or local glutamine supply. Therefore, future studies could measure the effects of other factors including hormones other than the ones used in the present study and BCAAs which could affect glutamine release from adipocytes isolated from these sites.

2. Glutamine metabolism in the omentum:

This study has shown that adipose tissue from the omentum has a high capacity to utilise and release glutamine. Given the close proximity between adipose tissue and lymphoid tissue in this depot, analysis of interactions between these two tissues may provide information on whether glutamine may be supplied preferentially for immune tissues in this depot.

3. The fate of glutamine in the adipocyte was not assessed in the present study. It would be of interest to measure the effects of different hormones on the fate of glutamine in different adipose depots to ascertain whether it may contribute significantly to *de novo* lipogenesis and possible hypertrophy of depots such as the omentum.

4. The data from the present study suggest that a substrate cycle of glutamine exists in adipose tissue. Therefore, measurement of the relative flux of glutamine in selected adipose depots may provide more information on the value of such a cycle for regulation of whole-body glutamine supply.

APPENDICES

Appendix I

Technique used to determine protein content of adipose tissue samples

Adipose tissue samples were homogenised in 5% trichloroacetic and placed on ice for 15 minutes to allow the protein to precipitate. The samples were then centrifuged at 400 g using a MSE Coolspin centrifuge for 10 minutes at 4°C and the resultant pellet washed 3 times with ethanol:ether (1:1) to extract the acid and remaining lipids. The pellet was dissolved in 1 ml of NaOH (0.1 M) and the protein content measured by the Bio-Rad protein assay as used by Bradford 1976.

Protein content in adipose tissue samples from different depots of the guinea-pig. Values are mean \pm SEM mg of protein per 1 g of whole adipose tissue (n = 20):

DEPOT	mg g ⁻¹
In front of forelimb	6.9 \pm 0.3
Behind forelimb	7.0 \pm 0.6
Interscapular	6.5 \pm 0.1
Inguinal	5.2 \pm 0.7
Omental (adjacent to spleen)	29.6 \pm 3.2
Omental (far from spleen)	8.6 \pm 0.5
Mesenteric	6.9 \pm 0.7
Perirenal	6.6 \pm 0.2
Epididymal	8.2 \pm 1.3
Cervical	8.8 \pm 0.4
Popliteal	7.4 \pm 0.4

Appendix II

Chemicals and enzymes

The following chemical and enzymes were obtained from:

Sigma Chemical Co., Poole, Dorset, England

Tris aminomethane, ethylene(diamine)tetraacetic acid (EDTA), L-glutamine, sodium glutamate, triethanolamine, hydrazine hydrate, imidazole, leucine, Dowex-2-chloride, Triton X-100, Collagenase (type II) (from *Clostridium histolyticum*), (bovine serum albumin (BSA fraction V, fatty acid free), ouabain, insulin (bovine), (-)-isoprenaline and dexamethasone acetate.

Boehringer Mannheim UK, Lewes, England

Nicotinamide-adenine dinucleotide (NAD), adenosine-5'-diphosphate (ADP), adenosine-5'-triphosphate (ATP), creatine phosphate, glutamate dehydrogenase, and creatine kinase.

Amersham International, Aylesbury, England

L-[¹⁴C(U)] glutamic acid (specific activity 50 μ Ci ml⁻¹) and L-[¹⁴C(U)] glutamine.

GIBCO BRL, Paisley, U.K.

Hanks balanced salt solution.

Fluka Chemika, Gillingham, Dorset

Di-'isononyl' phthalate.

Pharmacia

Optiphas HIsafe 3.

All other chemicals used were of analytical grade.

Appendix III

Guinea-pig diet

All diet materials were purchased from:

SDS Ltd. Waltham, Essex

Guinea-pig Labsure diet RGP standard pellet size, 4 mm.

Average calculated composition of diet:

Proximate analysis%		Amino Acids	g/kg
Ash	7.0	Threonine	6.7
Crude oil	3.4	Glycine	10.6
Crude protein	17.4	Valine	9.0
Crude fibre	8.8	Cystine	2.9
Calcium	1.0	Methionine	3.5
Phosphorus	0.78	Isoleucine	7.3
Sodium	0.47	Leucine	12.0
Potassium	1.19	Tyrosine	5.6
Magnesium	0.42	Phenylalanine	8.0
Chloride	0.73	Lysine	8.4
Carbohydrate	50	Histidine	3.9
Starch	27	Arginine	11.6
Digestible energy MJ/kg	11.6	Tryptophan	2.4

15% fat enriched diet was prepared by combining powdered RGP and Lard (85 g RGP/15 g lard) and adding sufficient water to bind the mixture. The mixture was placed in trays, compressed and left to dry for 48 hrs. Once dry, the 'cake' was broken into 2 to 3 cm pieces.

Appendix IV

Preparation and care of reagents.

Preparation of Dowex Formate for glutamine synthetase activity measurement

Approximately 250 g of Dowex-2-chloride (dry mesh 200-400) was washed until the supernatant was clear, using distilled water for the first wash and de-ionised water for subsequent washes. The resin was filtered (Whatman number 1 filter paper) using a Buchner funnel under suction then added to a flask containing 4 l NaOH (1 M) and mixed for 30 minutes to generate the hydroxylated form. After allowing the resin to settle, the NaOH was decanted off and replaced with 4 l of distilled water for further washing. This procedure was repeated at least 4 times to ensure that the resin was free of alkali. The hydroxylated Dowex was filtered using a Buchner funnel and Whatman number 1 filter paper then placed in a fume cupboard before adding 250 ml of formic acid solution (98%) which was left to percolate through the Dowex. The formatted Dowex was then washed at least 3 times using 1.5 l each wash of distilled water and stored at 4°C until use.

Preparation of hormones or their analogues:

Bovine insulin (27.3 units mg⁻¹) was dissolved in 0.01 M HCl to a final concentration of 10 units ml⁻¹. Aliquots of insulin (100 µl) were dispensed into Eppendorf tubes and stored at -20 °C. On the day of the experiment an aliquot was thawed at 4 °C, and serial dilutions were made using KRB to give final concentrations of 0.1 to 1000 µU ml⁻¹ to be used in the incubation procedure.

A stock solution of dexamethasone (2.5 mM) was prepared in EtOH (100%), 1 ml aliquots of dexamethasone were dispensed into Eppendorf tubes and stored at 4 °C for up to 1 week prior to the uptake experiments. On the day of the experiment the stock

dexamethasone was diluted with KRB to give final concentration range of 100 to 2500 μM .

Isoprenaline was dissolved in 0.1 M HCl to a final concentration of 1 mM. Aliquots of isoprenaline (200 μl) were dispensed into Eppendorf tubes and stored at -20°C . On the day of the experiment an aliquot was thawed at 4°C , and serial dilutions were made using KRB for final concentrations of 0.1 to 100 μM to be used in the incubation procedure.

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